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CANCER TREATMENT REPORTS

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* *From* Methodologic Guidelines for Reports of Clinical Trials (Cancer Treat Rep 69:1–3, 1985).

Contents

Cancer Treatment Reports
Volume 69, Number 6
June 1985

Clinical and Phase I–II Studies

571 Intensive Induction Chemotherapy for Small Cell Anaplastic Carcinoma of the Lung. **M. R. O'Donnell, J. C. Ruckdeschel, D. Baxter, M. F. McKneally, R. Caradonna, and J. Horton.** Albany Medical College, New York

577 Comparison of Methotrexate and Cisplatin for Patients With Advanced Squamous Cell Carcinoma of the Head and Neck Region: A Southwest Oncology Group Study. **W. E. Grose, D. E. Lehane, D. O. Dixon, W. S. Fletcher, and W. J. Stuckey.** Southwest Oncology Group

583 Treatment of Non-Small Cell Bronchogenic Carcinoma With Vinblastine and Mitomycin: A Southwest Oncology Group Study. **J. K. Weick, J. M. Rainey, R. B. Livingston, L. H. Baker, R. M. O'Bryan, and T. T. Chen.** Southwest Oncology Group

587 Combination Chemotherapy Followed by Radiation Therapy in Patients With Regional Stage III Unresectable Non-Small Cell Lung Cancer. **R. Fram, A. Skarin, J. Balikian, D. Amato, N. Leslie, A. Malcolm, and E. Frei, III.** Dana-Farber Cancer Institute, Boston, Massachusetts

591 Adenocarcinoma of Unknown Primary Origin: Treatment With Vindesine and Doxorubicin. **J. J. Fiore, D. P. Kelsen, R. J. Gralla, E. S. Casper, G. Magill, E. Cheng, and M. Ochoa, Jr.** Memorial Sloan-Kettering Cancer Center, New York, New York

595 Mathematical Model of Remission Duration in Acute Myelogenous Leukemia. **B. G. Birkhead.** University College London, England

603 High-Dose Etoposide in the Treatment of Relapsed Primary Brain Tumors. **G. P. Finn, T. Bozek, R. L. Souhami, M. L. Slevin, and D. G. T. Thomas.** University College Hospital, London, England

607 Phase I Study of Divided-Dose Vinblastine in Advanced Malignancy. **J. A. Young, D. Westlake, G. W. Schnetzer, III, A. M. Keller, J. M. Sexauer, and L. N. Newcomer.** Natalie Warren Bryant Cancer Center, St. Francis Hospital, and Warren Research Institute, Tulsa, Oklahoma

611 Phase I–II Clinical Trial of Thymidine, 5-FU, and PALA Given in Combination. **D. F. Chiuten, M. Valdivieso, A. Bedikian, G. P. Bodey, and E. J Freireich.** The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston

Clinical and Preclinical Pharmacology

615 Clonogenic and Nonclonogenic In Vitro Chemosensitivity Assays. **L. M. Weisenthal and M. E. Lippman.** Veterans Administration Medical Center, Long Beach, California, and University of California, Irvine

633 Comparative Pharmacokinetics and Metabolism of Doxorubicin and Epirubicin in Patients With Metastatic Breast Cancer. **J. Robert, P. Vrignaud, T. Nguyen-Ngoc, A. Iliadis, L. Mauriac, and P. Hurteloup.** Fondation Bergonié, Bordeaux, France

641 Competitive Protein-Binding Assay for Trimetrexate. **J. C. Drake, C. J. Allegra, G. A. Curt, and B. A. Chabner.** National Cancer Institute, Bethesda, Maryland

645 Relevance of the Pharmacology of Oral Tegafur to Its Use as a 5-FU Pro-drug. **J. E. Byfield, C. L. Hornbeck, S. S. Frankel, T. R. Sharp, and J. C. Griffiths.** University of California, San Diego

653 Metabolic Effects of Gallium Nitrate Administered by Prolonged Infusion. **R. P. Warrell, Jr, M. Isaacs, C. J. Coonley, N. W. Alcock, and R. S. Bockman.** Memorial Sloan-Kettering Cancer Center and Cornell University Medical College, New York, New York

657 Pharmacokinetics of Hexamethylmelamine Administered via the Ip Route in an Oil Emulsion Vehicle. **A. D. Wickes and S. B. Howell.** University of California-San Diego, La Jolla

663 Evidence for a Novel Hybrid Immunotoxin Recognizing Ricin A-Chain by One Antigen-Combining Site and a Prostate-Restricted Antigen by the Remaining Antigen-Combining Site: Potential for Immunotherapy. **K. S. Webb, J. L. Ware, S. F. Parks, P. J. Walther, and D. F. Paulson.** Duke University Medical Center, Durham, North Carolina

673 Promotion of Daunorubicin Uptake and Toxicity by the Calcium Antagonist Tiapamil and Its Analogs. **D. Kessel and C. Wilberding.** Wayne State University School of Medicine and Harper-Grace Hospitals, Detroit, Michigan

677 Increased Glutathione-*S*-Transferase Activity in a Cell Line With Acquired Resistance to Nitrogen Mustards. **A. L. Wang and K. D. Tew.** Georgetown University, Washington, DC

683 Stability of the In Vivo P388 Leukemia Model in Evaluation of Antitumor Activity of Natural Products. **J. C. Marsh, R. H. Shoemaker, and M. Suffness.** National Cancer Institute, Bethesda, Maryland

BRIEF REPORTS

687 Leukemic Meningitis in B-Cell Chronic Lymphocytic Leukemia. **J. P. Steinberg, M. Pecora, and J. L. Lokey.** Emory University School of Medicine, Atlanta, Georgia

689 Six-Drug Chemotherapy (Hexamethylmelamine, Doxorubicin, Cisplatin, Cyclophosphamide, Methotrexate, and 5-FU; CHAMP-5) for Ovarian Carcinoma: Alternating Sequences of Combination Regimens. **M. W. Pasmantier, M. Coleman, R. T. Silver, and W. P. Ballard.** The New York Hospital-Cornell University Medical Center, New York, New York

695 Cisplatin-Containing Chemotherapy in the Treatment of Invasive Thymoma: Report of Five Cases. **G. Giaccone, R. Musella, O. Bertetto, M. Donadio, and A. Calciati.** Ospedale S. Giovanni, Torino, Italy

699 Phase I Evaluation of β-2′-Deoxythioguanosine in Pediatric Patients With Leukemia. **G. R. Higgins, D. C. Jamin, N. A. Shore, R. Momparler, G. Hartman, and S. E. Siegel.** Childrens Hospital of Los Angeles and University of Southern California School of Medicine

703 Phase I Study of Bisantrene in Acute Nonlymphoblastic Leukemia. **M. Marty, C. Ferme, C. Gisselbrecht, H. Guy, M. J. Clark, A. Bancillon, and M. Boiron.** Hôpital Saint-Louis, Paris, France

707 Phase II Study of Elliptinium in Advanced Breast Cancer. **J. G. Rouësse, T. Le Chevalier, P. Caille, J. M. Mondesir, H. Sancho-Garnier, F. May-Levin, M. Spielmann, R. De Jager, and J. L. Amiel.** Institut Gustave-Roussy, Villejuif, France

709 Phase II Trial of Diaziquone in Advanced Large Bowel Carcinoma in Previously Treated and Untreated Patients: A Southwest Oncology Group Study. **R. Shildt, R. L. Stephens, V. P. Subramanian, L. H. Baker, W. S. Fletcher, R. M. O'Bryan, and J. D. McCracken.** Southwest Oncology Group

711 Phase II Trial of High-Dose Cisplatin in Patients With Malignant Mesothelioma. **D. M. Mintzer, D. Kelsen, D. Frimmer, R. Heelan, and R. Gralla.** Memorial Sloan-Kettering Cancer Center and Cornell University Medical College, New York, New York

713 Phase II Trials of Hexamethylmelamine, Dianhydrogalactitol, Razoxane, and β-2′-Deoxythioguanosine as Single Agents Against Advanced Measurable Tumors of the Pancreas. **Gastrointestinal Tumor Study Group**

717 Subrenal Capsule Assay: Feasibility of Transporting Tissues to a Central Facility for Testing. **D. E. Slagel, P. DeSimone, M. Dillon, D. J. LePage, and A. E. Bogden.** University of Kentucky Medical Center, Lexington

CLINICAL TRIALS SUMMARIES

719 Treatment of Refractory Hodgkin's Disease With Teniposide, Cisplatin, Hexamethylmelamine, and Prednisolone: Cancer and Leukemia Group B Trial 8171. **D. R. Budman, J. Anderson, J. P. Obrecht, G. P. Canellos, P. Creaven, L. Norton, and A. Gottlieb.** North Shore University Hospital and Cornell University Medical College, Manhasset, New York

721 Interferon and Doxorubicin in Renal Cell Carcinoma. **H. B. Muss, C. Welander, M. Caponera, K. Reavis, J. M. Cruz, M. R. Cooper, D. V. Jackson, Jr, F. Richards, II, J. J. Stuart, C. L. Spurr, D. R. White, P. J. Zekan, and R. L. Capizzi.** Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina

723 Phase II Study of Mitolactol in Advanced Malignant Melanoma. **N. Murray, H. Silver, A. Shah, and K. Wilson.** Cancer Control Agency of British Columbia and University of British Columbia, Vancouver, Canada

725 Phase II Study of Aclarubicin in Previously Untreated Patients With Advanced Soft Tissue Sarcoma: A Southeastern Cancer Study Group Trial. **M. Bertrand, P. Multhauf, A. Bartolucci, D. Ellison, and J. Gockerman.** Southeastern Cancer Study Group

727 Phase II Evaluation of Bisantrene in Patients With Advanced Renal Cell Carcinoma. **W. K. Evans, F. A. Shepherd, M. E. Blackstein, D. Osoba, and D. Taylor.** Toronto General Hospital, Mount Sinai Hospital and Toronto Bayview Cancer Clinic, Ontario, Canada

LETTERS

729 Effects of Cytotoxic Agents on Iron Metabolism. **M. Lane, C. P. Alfrey, Jr, and F. E. Smith.** Baylor College of Medicine, Houston, Texas

729 Reply. **V. S. Sethi, Z. Shihabi, and C. L. Spurr.** University of Texas at El Paso

730 Acute Cardiac Failure From Doxorubicin Cardiotoxicity Provoked in Relation to Second-Look Laparotomy. **K. Aabo, S. A. Mortensen, T. Jonsson, and H. H. Hansen.** The Finsen Institute, Copenhagen, Denmark

731 Successful Continuation of Cisplatin Therapy After Partial Recovery From Acute Renal Failure. **D. C. Wong and A. B. Deisseroth.** University of California at San Francisco

733 Combination of Lomustine and Cimetidine in the Treatment of a Patient With Malignant Glioblastoma: A Case Report. **W. A. Hess and P. L. Kornblith.** National Institutes of Health, Bethesda, Maryland

733 Acute ECG Changes During Cyclophosphamide Infusion in a Patient With Bronchogenic Carcinoma. **J. Shachor, B. Beker, Y. Geffen, and I. Bruderman.** Meir General Hospital, Kfar-Saba, Israel

735 Local Toxicity From Iv Administration of Baker's Antifol. **S. H. Krasnow, R. H. Patterson, B. Fischmann, M. A. Eisenberger, A. Johnston-Early, M. L. Citron, and M. H. Cohen.** Veterans Administration Medical Center, Washington, DC

737 ANNOUNCEMENTS

Intensive Induction Chemotherapy for Small Cell Anaplastic Carcinoma of the Lung[1,2,3]

Margaret R. O'Donnell,[4,5,6] John C. Ruckdeschel,[4,*] Donald Baxter,[4] Martin F. McKneally,[4] Richard Caradonna,[4] and John Horton[4]

The role of intensive induction chemotherapy in small cell cancer of the lung remains unclear. Twenty-eight newly diagnosed patients with small cell lung cancer were randomized to receive either high-dose or standard-dose cyclophosphamide, vincristine, and semustine in a trial where the dose of induction chemotherapy was the sole treatment variable. Complete responses (CR) were achieved in 57% of patients receiving high-dose therapy as compared to 21% of patients receiving standard-dose therapy ($P < 0.05$). There was a higher rate of severe toxicity in the high-dose group ($P < 0.01$). The overall median survival was not improved (36 weeks for the high-dose group vs 43 weeks for the standard-dose group); however, the median survival in patients achieving CR was prolonged (92 weeks for the high-dose group vs 50 weeks for the standard-dose group). These effects were most pronounced in patients with extensive disease; a CR after induction was achieved in five of nine patients treated with high doses but not in any of those treated with standard doses ($P < 0.05$). A small group of patients appear to benefit by achieving a long-term remission with intensive induction chemotherapy, but this effect may be offset by increased morbidity. [Cancer Treat Rep 69:571–575, 1985]

Both animal and human studies have shown evidence for increasing tumor response with dose escalation of alkylating agents such as cyclophosphamide (CTX) and nitrosoureas in a wide variety of tumors (1-4). At the time this trial was designed, the results of Cohen et al (5) suggested improved response rates and survival times for patients treated with intensive, as opposed to standard, doses of a similar regimen (CTX, lomustine, and methotrexate). The results of a pilot study evaluating the effectiveness of intensive induction chemotherapy comprised of high-dose CTX, vincristine, and semustine (methyl-CCNU) in inducing complete responses and in prolonging median survival times in small cell lung cancer when compared to the same drugs in the more conventional doses are reported here.

MATERIALS AND METHODS

Between June 1977 and April 1980, 28 patients with newly diagnosed, histologically confirmed small cell anaplastic lung carcinoma were randomly assigned to receive either intensive- or standard-dose induction chemotherapy. The extent of measurable or evaluable disease at diagnosis was documented with marrow aspirate and biopsy and radionuclide scans of bone, brain, and liver. Patients with brain metastases were excluded from the study. Renal and hepatic functions were adequate (BUN $\leqslant$ 30 mg/100 ml, creatinine $\leqslant$ 1.5 mg/100 ml, bilirubin $<$ 2.0 mg/100 ml, and SGOT $<$ 60 IU/ml) in all patients. Hematologic parameters were normal (wbc count $\geqslant 4000/\mu$l and platelet count $\geqslant 100{,}000/\mu$l) in 26 pa-

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[3] Presented at the 17th Meeting of the American Association for Cancer Research, Washington, DC, May 29, 1981.

[4] Division of Oncology (*M. R. O'Donnell, J. C. Ruckdeschel, R. Caradonna,* and *J. Horton*), Department of Radiation Therapy (*D. Baxter*), and Division of Cardiothoracic Surgery (*M. F. McKneally*), Albany Medical College, NY.

[5] Section of Medical Oncology, Albany Veterans Administration Medical Center, NY.

[6] *Present address:* Department of Hematology and Bone Marrow Transplantation, City of Hope National Medical Center, Duarte, CA.

[*] *Reprint requests to:* John C. Ruckdeschel, MD, Division of Oncology, Albany Medical College, Albany, NY 12208.

tients; the other two had extensive marrow involvement producing pancytopenia in one patient and disseminated intravascular coagulation with thrombocytopenia in the other. Patients were stratified by performance status [Eastern Cooperative Oncology Group (ECOG) 0-1 vs 2-3] and extent of tumor (limited vs extensive). All 13 patients with limited disease had tumor confined to one hemithorax, mediastinum, and/or ipsilateral supraclavicular node. Seven of 15 patients with extensive disease had multiple areas of systemic involvement; in the eight with a solitary extrathoracic site, bone was involved in five, and liver, skin, or cervical lymph node was involved in one each.

Induction treatment consisted of three cycles of either high-dose or standard-dose chemotherapy followed by irradiation to the mediastinum and residual thoracic tumor (fig 1). The high-dose induction was given every 3 weeks. Therapy was delayed for an additional week if the wbc count was < 2500/μl or the platelet count was < 50,000/μl; no subsequent dose modification was made for nadir cytopenias. The standard induction course was also given every 3 weeks, but dose modifications of 50% were made if the wbc count was 2500-4000/μl or the platelet count was 50,000-100,000/μl at the beginning of the next cycle. If the wbc or platelet counts were below these parameters, chemotherapy was delayed until the wbc and platelet counts reached these minimum levels. In all subsequent cycles CTX and methyl-CCNU were given at 50% doses.

Radiation therapy was started after hematologic recovery following Cycle 3 of the induction regimen. Patients received 3000 rads in ten 300-rad fractions to the residual primary tumor and mediastinum over a 2-week period. Prophylactic cranial irradiation was not administered since its role in improving survival was unproven at the time this study was begun and we wished to assess the possible role of methyl-CCNU in controlling subclinical brain metastases, as suggested by Donovan et al (6).

Maintenance chemotherapy began 2-3 weeks after completion of radiation therapy dependent on hematologic recovery, and was repeated every 4 weeks until relapse or until the total doxorubicin dose reached 540 mg/m^2. For the initial cycle after irradiation, doses were reduced by 50% to avoid possible radiation recall effects.

On completion of radiation therapy patients were evaluated for objective tumor response; patients with no clinical evidence of disease [complete response (CR)], were restaged with radionuclide scans, marrow biopsy, and bronchoscopy. Standard response criteria were used to define response and disease progression (7).

Statistics

Differences in survival and toxicity were determined by the Wilcoxon test and Kaplan-Meier curves; the Fisher exact test was used for evaluating induction response. The study was discontinued in 1980 because of the evidence that etoposide was an effective, front-line agent in this disease (8), that prophylactic cranial irradiation reduced the incidence of brain metastases and improved survival (9), and it was felt that consolidative thoracic irradiation was causing too lengthy a hiatus in chemotherapy for patients with initially extensive disease who achieved only a partial response (PR). Therefore, some groups contain too few patients for valid statistical evaluation.

RESULTS

Patient Characteristics

Patient group characteristics at time of randomization

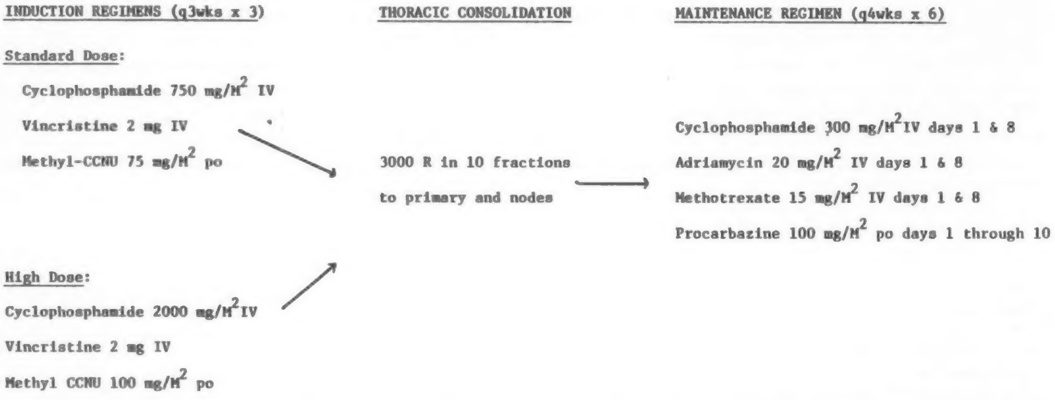
INDUCTION REGIMENS (q3wks x 3)
Standard Dose:
Cyclophosphamide 750 mg/M^2 IV
Vincristine 2 mg IV
Methyl-CCNU 75 mg/M^2 po
High Dose:
Cyclophosphamide 2000 mg/M^2 IV
Vincristine 2 mg IV
Methyl CCNU 100 mg/M^2 po
THORACIC CONSOLIDATION
3000 R in 10 fractions
to primary and nodes
MAINTENANCE REGIMEN (q4wks x 6)
Cyclophosphamide 300 mg/M^2 IV days 1 & 8
Adriamycin 20 mg/M^2 IV days 1 & 8
Methotrexate 15 mg/M^2 IV days 1 & 8
Procarbazine 100 mg/M^2 po days 1 through 10

FIGURE 1.—Induction treatment consisting of 3 cycles of either high-dose or standard-dose chemotherapy followed by irradiation to the mediastinum and residual thoracic tumor.

are shown in table 1. The groups are comparable except for slightly more females and patients with limited disease in the standard-dose group.

TABLE 1.—Patient characteristics*

Characteristic	Dose High	 Standard
No. of patients	14	14
Median age in yrs (range)	58 (46–66)	57 (44–77)
Male/female	13/1	10/4
Limited disease/ extensive disease	5/9	8/6
Performance status		
0–1	11	11
2–3	3	3
Pulmonary complications		
Superior vena cava obstruction	3	0
Obstructive pneumonia	2	5
Metastatic sites		
Bone	7	5
Liver	2	2
Marrow	1	2

* Unless otherwise specified, values = No. of patients.

Objective Tumor Response

After completion of induction chemotherapy, tumor regression (CR and PR) was observed in ten of 14 (73%) patients receiving high-dose treatment and in six of 14 (43%) receiving standard-dose treatment. When the groups were separated by extent of disease, three of five patients with limited disease receiving high-dose therapy achieved CR as compared to three of eight receiving standard-dose therapy ($P < 0.1$) (table 2). Among the patients with extensive disease, five of nine receiving high-dose treatment achieved CR, while none receiving standard-dose treatment achieved more than a PR prior to irradiation ($P < 0.05$). The responses were not limited to patients with solitary metastases; two of the four patients with multiple extrathoracic sites achieved CR.

Radiation therapy converted three patients with PR (two with limited disease and one with extensive disease) in the standard-dose group to CR status. Radiation therapy did not change response evaluation in any of the high-dose group.

Toxicity

Severe myelosuppression was expected with the high-dose induction course; all patients with leukocyte counts < 1000/μl and fever > 38°C were hospitalized in reverse isolation and treated with broad-spectrum antibiotics. Twenty-seven of 38 cycles of high-dose induction treatment had leukocyte count nadirs < 1500/μl; hospitalization for antibiotic therapy was required during 15 of the leukopenic episodes. Two patients with obstructive pneumonitis at diagnosis developed lung abscesses and a third developed empyema (10). There was one drug-related death due to erosion of an abscess cavity into the pulmonary artery.

Leukopenia was significantly less with standard induction therapy (table 3). Five of 39 cycles of standard-dose induction therapy resulted in severe leukopenia. Only one patient, who had severe marrow involvement at diagnosis, required hospitalization on two separate occasions, one for pneumonia and the second terminal admission for acute pancreatitis, bowel obstruction, and septicemia. Thrombocytopenia was a less frequent cause of hematologic toxicity. One patient in each group required platelet support. High-dose chemotherapy did not impair delivery of maintenance chemotherapy; ten high-dose patients received 74% of projected CTX, doxorubicin, methotrexate, and procarbazine maintenance as compared to 86% in eight standard-dose survivors. CNS metastases to epidural space or brain occurred during treatment in five patients in each group; the overall incidence of neurologic progression using methyl-CCNU as prophylaxis was 35%.

Survival

The overall median survival showed no significant difference between intensive induction therapy (36 weeks) and standard induction therapy (43 weeks) ($P > 0.1$) (fig

TABLE 2.—Incidence of objective tumor response after induction chemotherapy

Group	CR (%)	PR (%)	Disease progression (%)
High-dose			
Limited disease	3 (60)	1 (20)	1 (20)
Extensive disease	5 (56.1)	1 (11)	2 (22)
Total	8 (57.5)	2 (14)	3 (21.5)
Standard-dose			
Limited disease	3 (38)	2 (25)	1 (12)
Extensive disease	0 (0)	1 (16.7)	3 (50)
Total	3 (21.5)	3 (21.5)	4 (28.5)

TABLE 3.—Hematologic toxicity during induction

Group	Median wbc count/μl/cycle			Median platelet count/μl/cycle		
	1	2	3	1	2	3
High-dose	1400*	600*	400*	142,000	164,000	80,000†
Standard-dose	2900	3800	3600	187,000	214,000	210,000

*$P \leq 0.01$.
†$P \leq 0.05$.

2). The median survival for patients with limited disease in the standard treatment arm was 49 weeks and in the high-dose arm was 40 weeks.

In contrast, survival in patients with extensive disease was almost doubled with high-dose induction therapy (54 vs 28 weeks for standard dose). The difference reflects the high percentage of CRs in the high-dose group. The median survival for high-dose patients achieving CR was 92 weeks; two patients are alive, with no evidence of recurrent disease at 103+ and 206+ weeks. The median survival for standard-dose patients achieving CR was 50 weeks; no patients survived > 66 weeks.

DISCUSSION

Intensive induction therapy has produced high CR rates and prolonged survival times in high growth fraction tumors similar to small cell lung cancer. Both animal and human studies have shown evidence of increasing tumor response with escalating doses of alkylating agents such as CTX or the nitrosoureas. Looney et al (11) showed a twofold delay in tumor growth using rat hepatoma 392A when CTX was increased from 50 to 150 mg/kg. Similar dose-response results have been obtained for rat mammary tumor and Lewis lung carcinoma models (12).

In human studies one can also see a trend toward higher CR rates with increasing alkylator dose. Edmonson et al (13) reported a 3% CR rate using CTX at a dose of 1000 mg/m^2 iv every 3 weeks, while Ettinger et al (3) reported a 17% CR rate using a dose of 60 mg/kg iv on Days 1 and 2 every 4 weeks. Bunn et al (14), using two dosage schedules of CTX, methotrexate, and lomustine, reported similar improvement in response rates. In the lower dose schedule, the overall response rate was 56%, with a 0% CR rate; in the higher dose schedule, the overall response rate was 90%, with a 25% CR rate.

Our pilot study with high-dose CTX, vincristine, and methyl-CCNU does show a trend for more CRs and prolongation of survival in those patients achieving a CR. However, morbidity from hematologic toxicity was substantial and patterns of relapse demonstrated the lack of

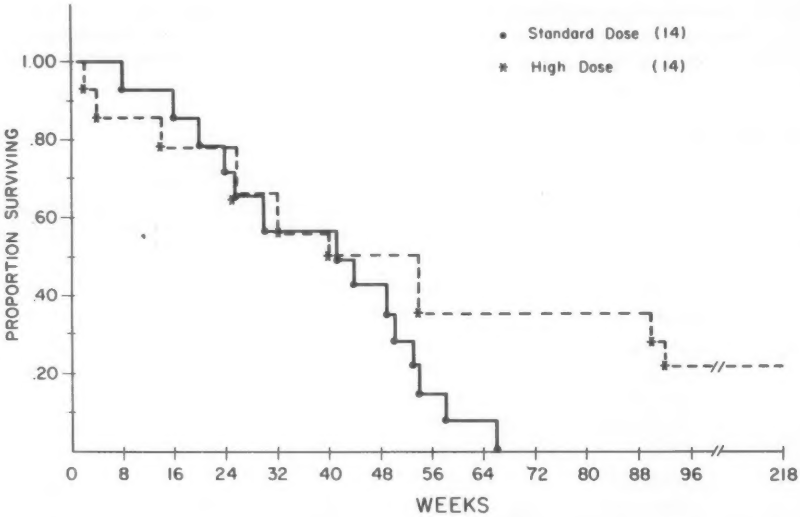

FIGURE 2.—Survival curves (Kaplan-Meier) of patients receiving high-dose induction therapy vs those receiving standard-dose induction therapy.

efficacy of methyl-CCNU in preventing CNS metastases when compared to prophylactic cranial irradiation (15,16). While this study did not demonstrate a clear-cut survival advantage for aggressive chemotherapy, a small percentage (15%) of patients are alive and disease-free > 2 years after initial treatment, a figure comparable to that currently obtained with other aggressive induction therapies (15), including those using autologous marrow rescue (15,17).

The ECOG tested the concept of high-dose induction therapy in a large randomized trial using a shorter induction course and somewhat lower doses of CTX and nitrosoureas as compared with our pilot (18). This study did show a survival advantage for patients with limited-stage disease [56 vs 42 weeks ($P = 0.02$)], but there was no significant improvement in overall survival and toxicity was clearly increased. Future trials need to identify subgroups of patients in whom the potential for improved CR rates and long-term survival overcomes the increased risk and mortality associated with high-dose induction chemotherapy.

REFERENCES

1. MUGGIA F, KREZASKI S, and HANSEN H. Cell kinetic studies in patients with small cell carcinoma of the lung. Cancer 34:1683-1690, 1974.
2. LEE R, CARR D, and CHILDS D. Comparison of split course radiation therapy and continuous radiation therapy for unresectable bronchogenic carcinoma: 5 year results. AJR Genl Rad Ther 126:116-127, 1976.
3. ETTINGER DS, KARP JE, ABELOFF MD, ET AL. Intermittent high-dose cyclophosphamide chemotherapy for small cell carcinoma of the lung. Cancer Treat Rep 62:413-424, 1978.
4. CORTES EP, TAKITA H, and HOLLAND JF. Adriamycin in advanced bronchogenic carcinoma. Cancer 34:518-524, 1974.
5. COHEN MH, CREAVEN PJ, FOSSIECK BE, JR, ET AL. Intensive chemotherapy of small cell bronchogenic carcinoma. Cancer Treat Rep 61:349-354, 1977.
6. DONOVAN T, BAXTER DS, and SPONZO R. The rationale for combined modality management in small cell carcinoma of the lung. Proc Am Assoc Cancer Res and ASCO 17:397, 1976.
7. OKEN MM, CREECH RH, TORMEY DC, ET AL. Toxicity and response criteria of the Eastern Cooperative Oncology Group. Am J Clin Oncol 5:649-655, 1982.
8. CAVALLI F, SONNTAG RW, JUNGI F, ET AL. VP-16-213 monotherapy for remission induction of small cell lung cancer: a randomized trial using three dosage schedules. Cancer Treat Rep 62:473-475, 1978.
9. SEYDEL HG, CREECH RH, MIETLOWSKI WL, ET AL. Preliminary report of a cooperative, randomized study for the treatment of localized small cell lung carcinoma. Int J Radiat Oncol Biol Phys 5:1445-1447, 1979.
10. BRANDMAN JR, RUCKDESCHEL JC, O'DONNELL MR, ET AL. Small cell cancer of lung. Rapid tumor necrosis leading to serious pulmonary infections after intensive chemotherapy. NY State J Med 9:1332-1334, 1981.
11. LOONEY WB, RITENOUR ER, and HOPKINS HA. Changes in growth rate of an experimental solid tumor following increasing doses of cyclophosphamide. Cancer Res 44:2179-2183, 1980.
12. MOORE JV, and DIXON B. The gross and cellular response of a rat mammary tumor to single doses of cyclophosphamide. Eur J Cancer 14:91-95, 1979.
13. EDMONSON JH, LAGAKOS SW, SELAWRY OS, ET AL. Cyclophosphamide and CCNU in the treatment of inoperable small cell carcinoma and adenocarcinoma of the lung. Cancer Treat Rep 60:925-932, 1976.
14. BUNN PA, JR, COHEN MH, IHDE DC, ET AL. Advances in small cell bronchogenic carcinoma. Cancer Treat Rep 61:333-342, 1977.
15. LIVINGSTON R. Small cell carcinoma of the lung. Blood 56:575-584, 1980.
16. HANSEN H. Should initial treatment of small cell carcinoma include systemic chemotherapy and brain irradiation? Cancer Chemother Rep Part 3, vol 4:239-241, 1973.
17. SPITZER G, FARHA P, DICKE K, ET AL. High dose intensification with autologous bone marrow transplantation in limited small cell bronchogenic carcinoma. Proc ASCO 25:221, 1984.
18. MEHTA C, and VOGL SE. High dose cyclophosphamide in the induction chemotherapy of small cell lung cancer—minor improvements in rate of remission and survival. Proc Am Assoc Cancer Res 23:155, 1982.

Comparison of Methotrexate and Cisplatin for Patients With Advanced Squamous Cell Carcinoma of the Head and Neck Region: A Southwest Oncology Group Study[1,2]

William E. Grose,[3] Daniel E. Lehane,[3] Dennis O. Dixon,[4] William S. Fletcher,[5] and W. J. Stuckey[6,*]

One hundred eligible patients with inoperable, locally advanced or metastatic squamous cell carcinoma of the head and neck region following prior therapy were randomized to receive im methotrexate (50 patients) or iv cisplatin (50 patients). Methotrexate produced a complete plus partial response rate of 16.0% and cisplatin produced a partial response rate of 8.0%, with median durations of response of 18 and 8 weeks, respectively. The corresponding median survival times were 20 and 18 weeks. Methotrexate responders survived 60 weeks, versus 17 weeks for nonresponders. The corresponding survival times for cisplatin-treated patients were 38 and 18 weeks. Good pretreatment performance status had a significantly positive effect on survival (*P* = 0.04), but prior therapy did not. Toxicity secondary to either agent occurred with the expected frequency. The two agents examined showed comparable antitumor activity in patients with squamous cell carcinoma of the head and neck who had not previously received chemotherapy. [Cancer Treat Rep 69:577–581, 1985]

Conventional low-dose methotrexate (MTX), prescribed as a single agent, is frequently regarded to be the most effective and the least toxic chemotherapeutic agent available for the treatment of advanced squamous cell carcinoma of the head and neck region. While many schedules examined clinically have been found to be equally effective, treatment every 3 weeks has been associated with an immunologic rebound which occurs between treatment cycles (1). The Southwest Oncology Group (SWOG) has completed a comparative phase III trial using this schedule effectively, administering methotrexate alone and in combination with semustime and bleomycin (2). The response rates were similar between the two regimens (about 30%), but the combination was more toxic. Similarly, the SWOG reported on the efficacy (about 20%) and toxicity (acceptable) of cisplatin (CACP) in previously treated patients with head and neck squamous cell cancer using a twice monthly outpatient program (3).

Since CACP had not been tested in patients who had not received prior chemotherapy, a phase III trial comparing CACP with the immunologically favorable MTX administration schedule was initiated in June 1978. This report describes the results of this randomized trial.

MATERIALS AND METHODS

Adult patients with histologically proven, recurrent, locally advanced, and/or metastatic squamous cell carcinoma of the head and neck region not amenable to other forms of therapy were eligible. All patients had measurable disease and had not previously received MTX or CACP. None of the patients had received antitumor therapy during the preceding 3 weeks. The patients were required to have a creatinine clearance of ⩾ 50 ml/minute, in addition to having an initial wbc count of at least 4000/mm^3 and a platelet count of at least 125,000/mm^3. Informed consent was obtained from each patient prior to therapy.

Patients were randomized to receive either MTX or CACP. MTX was administered at a dose of 15 mg/m^2 im daily for 3 consecutive days, repeated at 3-week inter-

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[3] Baylor College of Medicine, Houston TX.

[4] Southwest Oncology Group Biostatistical Center, Houston, TX.

[5] University of Oregon Health Sciences Center, Portland.

[6] Tulane University, New Orleans, LA.

[*] *Reprint requests to:* Southwest Oncology Group (SWOG-7814), Operations Office, 4450 Medical Dr, San Antonio, TX 78229.

vals. CACP was administered at a dose of 50 mg/m^2 iv on Days 1 and 8, repeated at 4-week intervals. Each dose of CACP was diluted in 1 L of 5% dextrose in one-half normal saline and infused over 1-4 hours. Prior to each dose, the creatinine clearance must have been > 50 ml/minute. In both treatment arms, documentation of the response status was required before each course of therapy was repeated.

Response to treatment and toxicity grading were defined according to the standard SWOG criteria (2). Performance status was estimated on a scale of 0-5, as defined by the Eastern Cooperative Oncology Group. Survival curves were estimated by the method of Kaplan and Meier (4). The two-tailed log-rank test (5) was used to assess the statistical significance of differences between survival curves.

RESULTS

Between June 1978 and October 1979, a total of 107 patients were registered on this study by 30 participating institutions. (Planned accrual was 96 evaluable patients in each arm. When an interim report suggested low likelihood of demonstrating superiority with CACP, accrual was discontinued.) Fifty-two patients were randomized to receive MTX, and the remaining 55 were randomized to receive CACP. There were two and five patients, respectively, in each arm who are excluded from this analysis: two were ineligible (one each with squamous cell carcinoma of the skin and esophagus); two declined therapy following registration and had no survival information; and three had no available data on response, survival, or toxicity. Thus, all seven patients are excluded from survival analysis. The remaining patients, 100 fully and partially evaluable (50 treated with MTX and 50 treated with CACP), constitute the subject of this report. All of these patients are included in the analysis of response and survival. There were three patients in each arm who received a single course of either MTX or CACP. Five of these six patients had an obvious increase of disease following therapy, and the remaining patient had no response in the face of grade 4 leukopenia from MTX.

The patient characteristics are shown in table 1, which displays the comparability of the two treatment groups. The distribution of patients according to the primary site is shown in table 2.

Overall, there were a total of 12 responders [complete response (CR) plus partial response (PR)], yielding a response rate of 12.0%. Separately, there were eight of 50 responders to MTX (three CR and five PR) and four of 50 responders to CACP (all PR), giving response rates of 16.0% and 8.0%, respectively (table 3). These results are not significantly different, and 95% confidence limits for the difference between the rates are -5% and 21%. Among 46 patients with initial performance status of 0-1, eight (17.4%) achieved a response to either MTX or CACP, while four (8.2%) of 49 patients with initial performance status of 2-4 achieved a response. As shown in table 1, the great majority of patients had had prior therapy of some type (primarily radiotherapy and/or surgery). Among 40 patients who received radiotherapy alone, four (10%) achieved a response to either MTX or CACP, while seven (13%) of 54 patients who received surgery with or without radiotherapy achieved a response.

The length of response to treatment and the survival duration from start of treatment are summarized in table 3. As can be seen, once a remission is achieved, there is a trend to suggest a more sustained response by MTX (median, 18 weeks; range, 5-85 weeks; two censored, 11 and 16 weeks) than by CACP (median, 8 weeks; range, 4-19 weeks; none censored).

The overall median survival from the start of treatment of the 100 fully and partially evaluable patients was 20 weeks. When the survival curves are plotted sepa-

TABLE 1.—Comparative patient characteristics (N = 100)*

Characteristic	MTX	CACP
Fully and partially evaluable	50 (50)	50 (50)
Sex		
Male	41 (82)	43 (86)
Female	9 (18)	7 (14)
Median age in yrs (range)	59 (43-80)	58 (26-78)
Pretreatment performance status†		
⩽ 1	25 (52)	21 (45)
> 1	23 (48)	26 (55)
Prior therapy‡		
Yes	47 (96)	47 (100)
No	2 (4)	0

*Unless otherwise specified, values = No. of patients (%).

†Five patients with unknown performance status are excluded.

‡Four patients with unknown prior therapy are excluded.

TABLE 2.—Distribution of 100 patients by primary site

Primary site	No. of patients (%) MTX (N = 50)	CACP (N = 50)
Oral cavity	12 (24)	12 (24)
Oropharynx	14 (28)	14 (28)
Larynx	11 (22)	9 (18)
Hypopharynx	6 (12)	4 (8)
Nasopharynx	5 (10)	7 (14)
Paranasal sinus	2 (4)	0
Other*	0	4 (8)

* One each of nasal cavity, ear, parotid gland, and unknown primary.

rately for each treatment group, the median survival times are similar (MTX, 20 weeks; CACP, 18 weeks). The estimated percentages of patients surviving 1 year are 18% for MTX and 16% for CACP; 95% confidence limits for the difference between these percentages are -13% and 17%. MTX responders had a median survival time of 60 weeks, versus 35 weeks for CACP responders (table 3). As anticipated, performance status demonstrated a significant influence on survival duration. The median survival for patients with a status of 0 or 1 was 24 weeks, compared with 14 weeks for those with worse status ($P = 0.04$).

The impact of the type of prior therapy (radiotherapy alone vs surgery with or without radiotherapy) on survival from the start of therapy was examined. When these two groups were compared, no difference in survival was detected, the median times being 20 and 18 weeks for those who received prior radiotherapy alone and for those who received prior surgery with or without radiotherapy, respectively.

Among the 100 patients evaluable for response, a total of 18 (18%) had distant metastases, primarily to the lung (66.7%). Seven patients received MTX, and the remainder received CACP. Only one of the 18 patients showed a response to therapy; this patient, with distant metastasis to the lung, had a PR with CACP.

There were no patients who began therapy who subsequently refused further therapy because of toxicity. The various toxicity types and their frequency of occurrence as graded are outlined in table 4. The most serious toxic effect encountered was a single MTX-related death from pneumonia with sepsis that occurred during an episode of severe leukopenia. In table 4, responders and the remaining treated patients are listed separately to permit examination of a possible correlation of biologic effect (eg, myelosuppression, stomatitis) with the outcome of therapy. A patient with more than one toxicity type is entered with that corresponding frequency in the table. Among the eight patients who responded to MTX, there were six who experienced toxicity types (biologic effect) of ⩾ grade 2 severity. Thus, six of eight responding patients (75%) experienced a biologic effect of this severity. Similarly, 20 of 42 nonresponding patients (48%) experienced a biologic effect of equal magnitude. As anticipated, MTX induced more stomatitis, while CACP induced more frequent nausea and vomiting and more nephrotoxicity. Overall, toxicity from either drug was acceptable and occurred with the expected frequency.

DISCUSSION

The response rate of 16% to MTX is lower than the 33% response rate achieved in a recent SWOG study

TABLE 3.—Treatment outcome

	MTX (N = 50)	CACP (N = 50)	*P* value
% response (CR + PR)	16	8	0.36
Median duration of response (wks)	18	8	0.12
Median survival from treatment start (wks)			
Overall	20	18	0.99
Responders	60	35	0.17
Nonresponders	17	18	—
Performance status (all patients)			
⩽ 1	24		0.04
> 1	14		

TABLE 4.—Toxic effects of MTX (M) and CACP (P) (N = 100)

Toxicity type	Toxicity grade—*									
	Unknown		0		1		2-4		5	
	M	P	M	P	M	P	M	P	M	P
			Responders†							
Wbc	13		25	50	13		38	50	13	
Platelet	13		75	50		50	13			
Stomatitis	25	100	13		25		38			
Renal	50		50			50		50		
Nausea and vomiting	100	25						75		
			Other treated patients‡							
Wbc	14	7	48	70	10	11	29	13		
Platelet	17	7	74	78	2	4	7	11		
Stomatitis	21	85	38	15	5		36			
Renal	79	11	17	57	5	22		11		
Nausea and vomiting	100	33		7		11		50		
Other										
Ototoxic	100	96								
Hyponatremia	100	98								
Diarrhea	100	98								

*Values = % of patients.

†Among the responders (CR + PR), 8 patients were treated with MTX and 4 were treated with CACP.

‡Among the nonresponders, 42 patients were treated with MTX and 46 were treated with CACP.

using the same dose schedule (2). This cannot be attributed to any obvious differences in the two patient populations or in their pretreatment characteristics (2,6). These SWOG response rates, in turn, are lower than what have been achieved in other reported series, even though somewhat different dose schedules were used (7-9). Similarly, the response rate of 8% to CACP is disappointingly low in this study as compared to another recent SWOG study (3), and to other clinical trials involving previously treated (10,11) and untreated (12) patients with head and neck cancer. The response rates reported in those studies have been ≥ 25%. The reason for these differences is not clear. The presence of distant metastasis has been regarded by some as having an adverse impact on response (2,9). However, the frequency of distant metastases encountered in either the MTX group (15.9%) or the CACP group (22.9%) is not different from other reported series (13,14), to perhaps account for our results.

Recently, Hong et al (15) have reported on a randomized trial of weekly iv MTX versus CACP, the latter as prescribed in our study. Their results showed no difference in response and survival between the two agents in patients with recurrent squamous cell carcinoma of the head and neck, but MTX was better tolerated. In our study, although the MTX- and CACP-induced remission rates were not significantly different, a remission induced by MTX appeared to be more durable than that induced by CACP. The median durations of response noted here are comparable to those previously reported by the SWOG, 17 weeks (CR plus PR) for MTX (2) and 10 weeks (PR) for CACP (3).

The most frequent CACP toxicity in this study was nausea and vomiting, recorded in about two-thirds of the patients. This morbid side effect has recently been controlled quite successfully by administering high-dose metoclopramide (16,17). This approach appears promising to make future CACP treatments more tolerable and acceptable to both patients and physicians.

This study continues to demonstrate acceptable toxicity and activity of single-agent MTX administered as an intermittent every 3-week program in the management of advanced squamous cell carcinoma of the head and neck region. The two agents appear to confer comparable benefits in patients with head and neck cancer who had not previously received chemotherapy. To date, therapeutic trials of single agents (MTX or CACP) versus combinations have not established a significant benefit from the combinations used (2,9,18). MTX may continue to enjoy the role as a "standard" in comparative clinical trials.

REFERENCES

1. LEHANE DE, LANE M, and WARDELL M. Immunocompetence of head and neck cancer patients receiving methotrexate (MTX) by two different schedules. Proc Am Assoc Cancer Res and ASCO 15:190, 1974.
2. LEHANE DE, LANE M, GAD-EL-MAWLA N, ET AL. A comparison of methotrexate with a three-drug combination methotrexate methyl-CCNU and bleomycin for patients with advanced squamous cell carcinoma of the head and neck region. A Southwest Oncology Group Study. In press.
3. PANETTIERE FJ, LEHANE D, FLETCHER WS, ET AL. Cis-platinum therapy of previously treated head and neck cancer. The Southwest Oncology Group's two-dose-per-month outpatient regimen. Med Pediatr Oncol 8:221-225, 1980.

4. KAPLAN EL, and MEIER P. Non-parametric estimation from incomplete observations. J Am Stat Soc 53:457-481, 1958.
5. PETO R, and PETO J. Asymptotically efficient rank invariant test procedures. J R Stat Soc 135:185-207, 1972.
6. AMER MH, AL-SARRAF M, and VAITKEVICIUS VK. Factors that affect response to chemotherapy and survival of patients with advanced head and neck cancer. Cancer 43:2202-2206, 1979.
7. LANE M, MOORE JE, III, LEVIN H, ET AL. Methotrexate therapy for squamous cell carcinoma of the head and neck: intermittent intravenous dose program. J Am Med Assoc 204:561-564, 1968.
8. CARTER SK. The chemotherapy of head and neck cancer. Semin Oncol 4:413-424, 1977.
9. DECONTI RC, and SCHOENFELD D. A randomized prospective comparison of intermittent methotrexate, methotrexate with leucovorin, and a methotrexate combination in head and neck cancer. Cancer 48:1061-1072, 1981.
10. WITTES RE, CVITKOVIC E, SHAH J, ET AL. *cis*-Dichlorodiammineplatinum(II) in the treatment of epidermoid carcinoma of the head and neck. Cancer Treat Rep 61:359-366, 1977.
11. SAKA K, RAZACK MS, and KALMINO I. Chemotherapy for advanced and recurrent squamous cell carcinoma of the head and neck region with high and low dose *cis*-diamminedichloroplatinum. Am J Surg 136:529-533, 1978.
12. WITTES R, HELLER H, RANDOLPH V, ET AL. *cis*-Dichlorodiammineplatinum(II)-based chemotherapy as initial treatment of advanced head and neck cancer. Cancer Treat Rep 63:1533-1538, 1979.
13. PROBERT JC, THOMPSON CW, and BAGSHAW MA. Patterns of spread of distant metastases in head and neck cancer. Cancer 33:127-133, 1974.
14. MERINO OR, LINDBERG RD, and FLETCHER GH. An analysis of distant metastases from squamous cell carcinoma of the upper respiratory and digestive tracts. Cancer 40:145-151, 1977.
15. HONG WK, SCHAEFER S, ISSELL B, ET AL. A prospective randomized trial of methotrexate versus cisplatin in the treatment of recurrent squamous cell carcinoma of the head and neck. Proc ASCO 1:202, 1982.
16. GRALLA RJ, SQUILLANTE A, INTRIQ LM, ET AL. High dose metoclopramide: effective antiemetic against cisplatin (DDP) in randomized trials vs placebo and prochlorperazine (PCP). Proc Am Assoc Cancer Res and ASCO 22:420, 1981.
17. STRUM SB, MCDERMED JE, OPFELL RW, ET AL. Metoclopramide (MCP): an effective antiemetic in the prevention of chemotherapy-induced nausea and vomiting. Proc ASCO 1:59,1982.
18. DAVIS S, and KESSLER W. Randomized comparison of *cis*-diammine-dichloroplatinum versus *cis*-diammine-dichloroplatinum, methotrexate and bleomycin in recurrent squamous cell carcinoma of the head and neck. Cancer Chemother Pharmacol 3:57, 1979.

Treatment of Non-Small Cell Bronchogenic Carcinoma With Vinblastine and Mitomycin: A Southwest Oncology Group Study[1,2]

James K. Weick,[3] John M. Rainey,[4] Robert B. Livingston,[5] Lawrence H. Baker,[6] Robert M. O'Bryan,[7] and T. Timothy Chen[8,*]

Forty-five patients with stage III M, non-small cell bronchogenic carcinoma were treated with vinblastine (1 mg/m^2 by iv bolus twice a day on 2 consecutive days) plus mitomycin (10 mg/m^2 on Day 1). This treatment was repeated at 3-week intervals for three courses. Consolidation therapy with doxorubicin and cisplatin at doses of 50 mg/m^2 each was administered to responders every 4 weeks for two courses, with subsequent vinblastine and mitomycin maintenance therapy every 6 weeks. Eleven partial remissions (24%) were achieved, with a median duration of remission of 16 weeks (range, 8–32) and a median survival of 19 weeks. No differences were seen in median duration of remission or survival by cell type or performance status. [Cancer Treat Rep 69:583–585, 1985]

Lung cancer continues to be a leading cause of cancer deaths in the United States (1). Because most non-small cell bronchogenic cancer (NSCBC) is inoperable at the time of diagnosis, there is a need for improved systemic therapy in this disease. Large numbers of trials have failed to resolve the question of whether chemotherapy either alone or in combination has improved survival. It is generally accepted that the important prognostic features in NSCBC include stage of disease, performance status (PS), and weight loss (2). The objective response rates to most single chemotherapeutic agents employed in lung cancer are between 5% and 35% (3-10). These responses are generally short and a rare complete response is noted. The greatest activity has been reported for anthracycline derivatives, mitomycin, cisplatin, vinca alkaloids, and etoposide (4-10). Two recent reports by Bhaskar and Schulman et al (11-13) used vinblastine with or without mitomycin with reports of complete responders, minimum toxicity, and prolonged survival. Comparison pharmacokinetics of the vinca alkaloids has demonstrated high serum clearance rates for vinblastine, suggesting that multiple dosing or infusion might be preferable to bolus injection (14). Because of the ease of administration of the agents vinblastine and mitomycin in the outpatient department, the Southwest Oncology Group has used this induction approach in a pilot study for extensive NSCBC.

METHODS AND MATERIALS

Patients were eligible for study with stage III M,, histologically proven adenocarcinoma, large cell carcinoma, or squamous cell carcinoma provided that an evaluable lesion was present. Metastatic disease to the ipsilateral hilar lymph node and/or mediastinal nodes *only* was not eligible. A previously radiated lesion was allowed as measurable disease only if there was documented growth. A PS of at least 30% (Karnofsky) was required. No prior chemotherapy was permitted and pretreatment requirements included wbc count $> 3000/\mu l$, platelet count $> 100{,}000/\mu l$, serum creatinine < 1.2 mg/dl, and serum bilirubin < 3.0 mg/dl. Vinblastine was administered at 1.0 mg/m^2 by iv bolus given in the AM and PM of 2 consecutive days. Mitomycin was given at 10 mg/m^2 by iv bolus on Day 1. All medications were administered on an outpatient basis. This treatment was repeated at 3-week intervals, for a total of three courses. Patients with no improvement at this time were then removed from the

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[3] Cleveland Clinic Foundation, OH.

[4] Louisiana State University Medical Center, New Orleans.

[5] Puget Sound Oncology Consortium, Seattle, WA.

[6] Wayne State University Medical Center, Detroit, MI.

[7] Henry Ford Hospital, Detroit, MI.

[8] Southwest Oncology Group Statistical Office, Houston, TX.

[*] *Reprint requests to:* Southwest Oncology Group (SWOG-8260), Operations Office, 4450 Medical Dr, San Antonio, TX 78229.

study. Patients with an evaluable response or stable disease received inpatient consolidation chemotherapy as follows: doxorubicin (50 mg/m^2 iv on Day 1 of Weeks 13 and 17) and cisplatin (50 mg/m^2 on Day 1 of Weeks 13 and 17) (15). If disease progression was noted while the patient was receiving consolidation chemotherapy, reinduction therapy was attempted with vinblastine and mitomycin every 3 weeks for three courses. Failure of reinduction therapy thus removed the patient from the study. Maintenance chemotherapy consisted of vinblastine and mitomycin at induction dose levels at 6-week intervals.

On the day chemotherapy was due, if the wbc count was $< 3000/\mu l$ and the platelet count was $< 100{,}000/\mu l$, the next treatment course was withheld at least 1 week or until counts recovered to the pretreatment values. If the creatinine rose to > 1.5 mg/dl, no therapy was administered until the cause was adequately evaluated. If the bilirubin level was 1.0-3.0 mg/dl, the dose of vinblastine was reduced by 25%; if the bilirubin was > 3.0 mg/dl, the vinblastine was reduced by 50%.

Complete remission was defined as the disappearance of all clinical evidence of active tumor for a minimum of 4 weeks; partial remission was defined as a $\geqslant 50\%$ decrease in the sum of the products of bidimensional diameters of measured lesions. For patients with liver metastases, it was required to have a 30% reduction in the sum of measurements below the costal margin, also for a minimum of 4 weeks. Survival curves and response durations were evaluated by the method of Kaplan and Meier and the generalized Wilcoxon test (16,17). An adequate trial was defined as one course of treatment. Patients were removed from study who failed to achieve either a complete remission, partial remission, or stable disease after at least two courses of therapy or earlier progressive disease occurred after an adequate trial.

RESULTS

Forty-five evaluable patients were entered in this Southwest Oncology Group (SWOG) trial No. 8260 between May 1982 and September 1983, including 29 males and 16 females with the following histology: 21 with adenocarcinoma, 17 with squamous cell carcinoma, and seven with large cell carcinoma. Prior radiation therapy had been administered to 14 patients and 31 patients had received no prior therapy. A SWOG PS of 0-1 (Karnofsky, 100%-70%) was noted in 20 patients, with a SWOG performance status of 2 or 3 noted in 25 patients (Karnofsky, 60%-30%). A total of 128 courses of vinblastine and mitomycin were administered to the 45 patients (range, one to seven courses); 16 patients received 40 courses of consolidation therapy with doxorubicin and cisplatin.

Eleven partial remissions (24%) were achieved in the study group. In addition, 11 patients had objective improvement of evaluable but not measurable disease. The remission duration for the 11 responding patients was 16 weeks (range, 8-32). The median duration of remission by cell type was 9 weeks for adenocarcinoma, 15 weeks for squamous cell carcinoma, and 13 weeks for large cell carcinoma ($P = 0.63$; three samples). The median duration of remission was 19 weeks in eight patients with a PS of 0-1 and 8 weeks in three patients with a PS of 2-4.

The median survival for all patients was 19 weeks. By cell type, there was no significant difference in survival; for adenocarcinoma, squamous cell carcinoma, and large cell carcinoma survival times were 20, 13, and 16 weeks, respectively ($P = 0.72$; three samples). Comparison of survival by PS also showed no difference, with a median survival of 23 weeks in those patients with a PS of 0-1 versus 17 weeks in those with a PS of 2-4.

The program, as administered, was very well tolerated, with toxicity being myelosuppression and gastrointestinal only. The study design did not require weekly blood cell counts but only counts at the time of the succeeding treatment. Three patients had granulocyte counts $< 3000/\mu l$ and four had platelet counts $< 100{,}000/\mu l$. At the time of subsequent therapy, the median wbc count was 6.1×10^9/L (range, 2.2-11.8) and the median platelet count was 246×10^9/L (range, 39-672). Nausea and vomiting were mentioned as a minimum side effect in 11 patients. No elevations of serum creatinine were noted. No other toxic effects related to the drugs were mentioned.

DISCUSSION

The combination of vinblastine and mitomycin, as delivered in this pilot study of the SWOG, shows activity similar to that reported for the individual agents and is not additive in benefit. The time to response was $\leqslant 6$ weeks in all responders and this response was irrespective of hematologic or gastrointestinal toxic effects. No correlation was seen between the response rates and survival data and toxicity. The role of additional therapy with doxorubicin and cisplatin was very minimal; no patient with stable disease achieved a remission with the addition of these two agents. We were not able to confirm the earlier reports of improved survival with the combination of vinblastine and mitomycin. The low response rates and survival times underscore the need for continued search of more effective chemotherapy agents.

REFERENCES

1. SILVERBERG E. Cancer statistics 1983. CA 33:9-25, 1983.
2. AISNER J, and HANSEN HH. Commentary: Current status of chemotherapy for non-small cell lung cancer. Cancer Treat Rep 65:979-986, 1981.
3. LIVINGSTON RB. Combination chemotherapy of bronchogenic carcinoma. I. Non-oat cell. Cancer Treat Rev 4:153-156, 1977.
4. EAGAN RT, INGLE JN, CREAGAN ET, ET AL. VP-16-213 chemothera-

py for advanced squamous cell carcinoma and adenocarcinoma of the lung. Cancer Treat Rep 62:843-844, 1978.

5. GRALLA RJ, RAPHAEL BG, GOLBEY RB, ET AL. Phase II evaluation of vindesine in patients with non-small cell carcinoma of the lung. Cancer Treat Rep 63:1343-1346, 1979.
6. GRALLA RJ, CVITKOVIC E, and GOLBEY RB. *cis*-Dichlorodiammineplatinum(II) in non-small cell carcinoma of the lung. Cancer Treat Rep 63:1585-1588, 1979.
7. ITRI LM, GRALLA RJ, CHAPMAN RA, ET AL. Phase II trial of VP-16-213 in non-small-cell lung lancer. Am J Clin Oncol 5:45-47, 1982.
8. LUEDKE SL, LUEDKE DW, PETRUSKA P, ET AL. Vindesine (VDS) monochemotherapy for non-small cell lung cancer: a report of 45 cases. Cancer Treat Rep 66:1409-1411, 1982.
9. SAMSON MK, FRAILE RJ, LEICHMAN LP, ET AL. Clinical studies of mitomycin C in advanced adenocarcinoma of the lung. *In* Mitomycin C: Current Status and New Developments (Carter S, and Crooke S, eds). New York, Academic Press, Inc, 1979, pp 121-127.
10. SELAWRY OS. Response of bronchogenic carcinoma to adriamycin (NSC-123127). Cancer Chemother Rep Part 3, vol 6(2):349-351, 1975.
11. BHASKAR BS. Phase II study of vinblastine and mitomycin-C in advanced adenocarcinoma and undifferentiated large cell carcinoma of the lung. Proc Am Assoc Cancer Res and ASCO 20:229, 1978.
12. SCHULMAN P, BUDMAN DR, VINCIGUERRA V, ET AL. Phase I evaluation of divided-dose vinblastine sulfate. Cancer Chemother Pharmacol 5:67-69, 1980.
13. SCHULMAN P, BUDMAN DR, VINCIGUERRA V, ET AL. Phase II study of divided-dose vinblastine in non-small cell bronchogenic carcinoma. Cancer Treat Rep 66:171-172, 1982.
14. NELSON RL, DYKE RW, and ROOT MA. Comparative pharmacokinetics of vindesine, vincristine and vinblastine in patients with cancer. Cancer Treat Rev 7(suppl 1):17-24, 1980.
15. VOGL S, OHNUMA T, PERLOFF M, ET AL. Combination chemotherapy with adriamycin and *cis*-diamminedichloroplatinum in patients with neoplastic diseases. Cancer 38:21-26, 1976.
16. KAPLAN EL, and MEIER P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457-481, 1958.
17. GEHAN EA. A generalized Wilcoxon test for comparing arbitrarily singly-censored samples. Biometrika 52:203-223, 1965.

Combination Chemotherapy Followed by Radiation Therapy in Patients With Regional Stage III Unresectable Non-Small Cell Lung Cancer[1]

Robert Fram,[2,3] Arthur Skarin,[2,*] Jerry Balikian,[2,3] David Amato,[2] Nancy Leslie,[2] Arnold Malcolm,[2,4] and Emil Frei, III[2]

A chemotherapy combination of cyclophosphamide, doxorubicin, and cisplatin (CAP) was administered to 30 patients with stage III M_0 or M_1 (supraclavicular nodes) unresectable non-small cell lung cancer before and after radiotherapy. All patients had mediastinal metastases and most had T_2 or T_3 primary lesions. The response rate (complete plus partial) after two cycles of CAP was 47%, which increased to 66% (24% complete response rate) following radiotherapy. The overall median survival from initiation of chemotherapy was 9 months. CNS relapse occurred in five (26%) of 19 responding patients who did not receive prophylactic cranial irradiation in the early part of the study. [Cancer Treat Rep 69:587–590, 1985]

The survival rate of patients with stage III N_2M_0 non-small cell lung cancer (NSCLC) is poor, rarely exceeding 10%–15% despite radiation therapy (1). With recent combination chemotherapy programs, significant objective response rates have been achieved in metastatic lung cancer. The combination of cyclophosphamide, doxorubicin, and cisplatin (CAP) demonstrated a response rate of 39%–42% in patients with advanced NSCLC (2–3). Furthermore, in the Mayo Clinic study (4), the use of CAP concurrently with radiation therapy showed an improvement in survival compared to cyclophosphamide, doxorubicin, dacarbazine, and radiotherapy. In 1979, a series of studies was initiated at our institution using CAP in patients with extensive metastatic (M_1) NSCLC, in those with borderline (marginally) resectable stage III M_0, and in those with categorically unresectable stage III M_0 or M_1 (supraclavicular nodes) disease as defined by the authors. In the latter two groups of patients, CAP was employed prior to radiation therapy with or without subsequent surgery. Those patients initially responding to CAP received subsequent chemotherapy after completion of radiotherapy. The overall goals were to improve local control and reduce systemic micrometastases, thereby hopefully improving survival. Preliminary results were published for patients with borderline resectable disease (5). This article reports the results of CAP chemotherapy and radiotherapy in patients with categorically unresectable disease, ie, mediastinal metastases (N_2) without (M_0) or with (M_1) regional supraclavicular metastases.

MATERIALS AND METHODS

Thirty-two patients with histologically proven stage III M_0 or M_1 (supraclavicular nodes) NSCLC were entered into the study from 1979 to 1983. Thirty patients were evaluable; one patient was found to have metastatic disease at the initiation of therapy, and follow-up on a second patient was inadequate. All patients had Eastern Cooperative Oncology Group performance scores of < 2 and, with the exception of one patient, none had a pleural effusion at presentation. Informed consent was obtained from all patients prior to therapy, in accordance with the regulations of the Human Protection Committee at the Dana-Farber Cancer Institute. All patients had bronchoscopy, except for five with supraclavicular lymph node involvement who had node biopsy only. Nineteen patients had mediastinoscopy and two had left anterior mediastinotomy. Four patients had extensive mediastinal involvement on the basis of computerized tomographic scan exam-

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[2] Departments of Medical Oncology, Medicine, Oncoradiology, and Biostatistics, Dana-Farber Cancer Institute; The Joint Center for Radiation Therapy; and Harvard Medical School, Boston, MA.

[3] *Present address:* Division of Oncology, University of Massachusetts Medical Center, Worcester.

[4] *Present address:* Radiation Oncology, Vanderbilt University Hospital, Nashville, TN.

[*] *Reprint requests to:* Arthur T. Skarin, MD, Dana-Farber Cancer Institute, 44 Binney St, Boston, MA 02115.

ination. These patients were excluded from further invasive evaluation in view of the presence of a T_3 lesion with recurrent laryngeal nerve involvement (one patient), a T_3 lesion with invasion of the trachea and posterior mediastinum (one), extensive mediastinal involvement characterized by the presence of a locally invasive 8-cm mass (one), and mediastinal involvement in association with a pleural effusion (one). Patients were thus classified by the authors as categorically unresectable prior to therapy if they had bilateral or extensive nodal and/or extranodal mediastinal involvement (N_2); a T_3 lesion with extensive invasion of the chest wall, diaphragm, mediastinum, or visceral structures; or ipsilateral supraclavicular lymph node involvement (M_1). No patients had Pancoast's tumor. Patients with distant metastases beyond the primary site and not encompassed by one radiation port were excluded from the study. Patients in this study contrast with those considered by the authors as having borderline resectable stage III M_0 disease. The latter patients included those with T_3 lesions minimally invading local structures, including Pancoast's tumors, and those with unilateral intranodal mediastinal disease (N_2), who are considered elsewhere (5). Therapy consisted of two cycles of CAP chemotherapy with cyclophosphamide (500 mg/m^2), doxorubicin (50 mg/m^2), and cisplatin (50 mg/m^2), given iv every 3 weeks. Those patients responding to chemotherapy received split-course radiation therapy consisting of 3000 rad followed by a 2- to 2½-week rest period, a further 2500-2700 rad, and then four more cycles of CAP chemotherapy. Radiation dosage was 1000-1200 rad per week and fields initially consisted of AP-PA portals to cover the primary tumor volume and regional lymph nodes. The secondary or "cone down" field was via obliques to avoid excessive spinal cord dose and encompassed the primary tumor volume and mediastinum. Those patients not initially responding to chemotherapy received definitive thoracic irradiation consisting of 5500-6000 rad given to the primary tumor volume and draining regional lymph nodes, but no additional CAP chemotherapy. After June 1982, all patients received prophylactic cranial irradiation (3600 rad in 18 fractions) in view of the high rate of relapse in the brain.

The response to therapy was assessed after completion of the initial two cycles of CAP chemotherapy and again after completion of radiation therapy. A complete remission (CR) was defined as the disappearance of all measurable lesions for a minimum of 1 month. A partial remission (PR) consisted of a $\geqslant 50\%$ reduction in the sum of the products of the greatest tumor diameter and its perpendicular with a duration of at least 1 month. A minimal response was defined as an unequivocal but $< 50\%$ reduction in these parameters. Survival was assessed by the method of Kaplan and Meier (6).

RESULTS

The clinical characteristics of 30 evaluable patients are shown in table 1. All patients had mediastinal metastases and an additional six had T_3 lesions. Six had supraclavicular lymph node involvement (M_1). Important characteristics of this patient population also include the high frequency of adenocarcinoma, present in 57% of the patients.

The response rate (CR plus PR) after two cycles of chemotherapy was 47%, which increased to 60% if the four patients with regression of $< 50\%$ are included (table 2). Following radiotherapy, 66% of the patients had evidence of objective disease regression. Among the 13 patients with PR, radiation therapy resulted in five with CR and six with no further disease regression, while two had disease progression within the radiotherapy port. Also, two patients with a marginal response benefited from radiotherapy, achieving PR and CR. Radiotherapy in 12 patients showing no response to initial CAP resulted in five with PR, but seven showed no disease regression.

Twenty-eight of 30 patients have relapsed or progressed after therapy within 1-24 months (median, 2). To assess the causes underlying treatment failure, both the sites of recurrence and/or progressive disease, as well as the extent of therapy, were evaluated. In 19 patients with CR or PR after chemotherapy and thoracic radiation therapy, subsequent brain metastases occurred in five (26%). Prophylactic cranial irradiation was subsequently added to the protocol in 1982, and none of the final four patients have recurred at this site. The importance of the brain as a site for treatment failure is further emphasized by the lack of other sites of recurrent disease in two of five patients who responded to chemotherapy and radiation therapy, but subsequently recurred solely in the brain. The pathology of all patients with CNS relapses was remarkable,

TABLE 1.—Clinical characteristics

Characteristics	No. of patients
Evaluable	30
Median age in yrs (range): 57 (31-70)	
Male/female	21/9
Histology	
Squamous cell carcinoma	6
Adenocarcinoma	17
Large cell carcinoma	7
Stage*	
$T_1N_2M_0$	3
$T_2N_2M_0$	15
$T_2N_2M_1$†	5
$T_3N_2M_0$	6
$T_3N_2M_1$†	1

*American Joint Commission.

†M_1 = supraclavicular nodes.

TABLE 2.—Response to therapy

Response*	No. of patients— Responding after 2 cycles of CAP			No. of patients— Responding after radiation therapy†	
CR	1	47%	60%	7 (1 CR, 5 PR, 1 MR)	66%
PR	13			12 (6 PR, 1 MR, 5 NR)	
MR	4				
NR	12			10 (2 PR, 1 MR, 7 NR)	
Total	30			29	

*MR = marginal response; NR = no response.

†The response of patients after radiation therapy is listed with the response after chemotherapy shown in parentheses. One patient died after CAP chemotherapy prior to radiation therapy because of progressive carcinoma.

in that adenocarcinoma was present in five patients and large cell carcinoma in two. Recurrent or persistent regional disease was more notable in nonresponders and in those with marginal response after initial chemotherapy and radiotherapy, occurring in eight of ten patients (80%) compared to five of 19 patients (26%) who achieved CR or PR.

While 14 of 18 patients who responded to chemotherapy subsequently received adequate radiation therapy, three received < 5500 rad and a fourth patient did not receive further thoracic radiation upon deteriorating clinically with brain metastases. Four of 12 patients not responding after chemotherapy received < 5500 rad of thoracic irradiation. The reasons for giving less irradiation were progressive disease while receiving radiation therapy (one patient), clinical deterioration without demonstrable metastases (two), and treatment error (one). The radiation therapy received by the responding and nonresponding groups did not differ to any great extent.

The median survival of all patients from initiation of chemotherapy was 9 months. Those patients with CR or PR after two cycles of chemotherapy had a median survival of 13 months, while the median survival of those with either a minimal or no response was 6 months. This difference in survival does not necessarily imply that CAP chemotherapy prolongs survival; it only indicates that those who respond to CAP have a more favorable subsequent prognosis than those who do not respond (7). Owing to the small size of the study, the effect of achieving CR with radiotherapy on survival cannot be assessed. Toxicity consisted of esophagitis requiring treatment break or hospitalization (three patients); radiation pneumonitis (three); severe myelosuppression requiring transfusion or platelets, or neutropenia associated with sepsis (three); intractable nausea poorly responsive to iv indication (five); and ototoxicity (one). The combination of CAP and irradiation did not cause an unacceptable degree of acute esophagitis or cardiomyopathy as might be anticipated in combining a doxorubicin-containing regimen with radiation therapy.

Probably contributing to the poor treatment outcome was the inability to complete adjuvant CAP chemotherapy after radiotherapy in five of the 18 patients who initially responded to chemotherapy. Among these patients, CAP chemotherapy was not completed because of clinical deterioration resulting from brain metastases (two patients), progressive lung metastases (one), and weakness and anorexia (two).

DISCUSSION

In the present study, the response rate of 47% to CAP chemotherapy is somewhat higher than the 39% regression rate initially reported by Eagan et al (2), and considerably higher than a 22% response rate reported for patients with extensive disease (8). The lower response rate in the latter study is likely a result of a more extensive tumor burden, since only patients with metastatic disease were treated. With the addition of radiotherapy to CAP chemotherapy in limited thoracic disease, a response rate of 59% was subsequently reported (8), which is similar to the overall response of 66% achieved in the present study after radiation therapy. A median survival of 16 months in the Mayo Clinic study, however, exceeds the 9 months observed in the present study. Our survival results are comparable to previously reported survivals after radiation therapy only (1). Our patient population, however, differs with respect to the high percent of patients with adenocarcinoma (57%), a poor prognostic feature (9), as well as with respect to their advanced stage of disease, including patients with extensive mediastinal involvement as well as T_3 lesions and regional supraclavicular lymph node involvement (M_1). The survival of these patients was longer, however, than the previously reported 3 months for untreated patients with stage III disease (10). Furthermore, while a study of this size does not permit statistically valid assessments, regional recurrences appear substantially diminished in those patients initially responding to chemotherapy. The inability of some of our patients to tolerate chemotherapy after radiation therapy suggests that further cycles of chemotherapy prior to thoracic irradiation in patients who have responses may prove beneficial.

The occurrence of brain metastases as the only site of metastatic disease in two patients responding to chemotherapy and radiation therapy emphasizes the importance of employing prophylactic cranial irradiation in patients with a history of adenocarcinoma or large cell carcinoma and stage III M_0 unresectable disease (9,11). In a recent randomized study, prophylactic brain irradiation reduced the incidence of brain relapse from 13 of 48 patients (27) to two of 44 patients (5%) (11).

The synergistic effect of cisplatin with cyclophosphamide and doxorubicin has been previously reported (12,13). Furthermore, it is suggested that cisplatin demonstrates a dose-dependent cytotoxic effect by a recent study showing a superior duration of response using a combination of vindesine and cisplatin at 120 mg/m^2, compared to the same combination except with cisplatin at 60 mg/m^2; the median durations of response were 12 and 5.5 months, respectively (14). Higher doses of cisplatin with other agents may prove to be beneficial in future studies.

Despite modern radiation therapy, the prognosis of stage III M_0 or M_1 (supraclavicular nodes) lung cancer remains poor. The goals of employing chemotherapy in such patients, to increase local control and reduce systemic micrometastases, await the development of more active agents and more effective combination chemotherapy programs.

REFERENCES

1. HELLMAN S, KLIGERMAN MD, VON ESSEN CF, ET AL. Sequelae of radical radiotherapy of carcinoma of the lung. Radiology 82:1055, 1984.
2. EAGAN RT, INGLE JN, FRYTAK S, ET AL. Platinum-based polychemotherapy versus dianhydrogalactitol in advanced non-small cell lung cancer. Cancer Treat Rep 61:1339-1345, 1977.
3. BRITELL JC, EAGAN RT, INGLE JN, ET AL. *cis*-Dichlorodiammineplatinum(II) alone followed by adriamycin plus cyclophosphamide at progression versus *cis*-dichlorodiammineplatinum(II), adriamycin, and cyclophosphamide in combination for adenocarcinoma of the lung. Cancer Treat Rep 62:1207-1210, 1978.
4. EAGAN RT, LEE RE, FRYTAK S, ET AL. Randomized trial of thoracic irradiation plus combination chemotherapy for unresectable adenocarcinoma and large cell carcinoma of the lung. Int J Radiat Oncol Biol Phys 5:1401-1405, 1979.
5. SKARIN A, VEEDER M, MALCOLM A, ET AL. Chemotherapy (CAP) prior to radiotherapy (RT) and surgery in marginally resectable non small cell lung cancer (NSCLC). Proc ASCO 1:143, 1982.
6. KAPLAN EL, and MEIER P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457-458, 1958.
7. ANDERSON JR, CAIN KC, and GELBER RD. Analysis of survival by tumor response. Clin Oncol 1:710-719, 1983.
8. RUDOLPH A, LAWSON R, HARVEY H, ET AL. Cytoxan, etoposide, and cisplatin (CEP) versus cytoxan, adriamycin, and cisplatin (CAP) in the treatment of non small cell lung carcinoma (NSCLC). Proc ASCO 3:210, 1984.
9. COX JD, and YESNER RA. Adenocarcinoma of the lung: recent results from the Veterans Administration Lung Group. Am Rev Respir Dis 120:1025-1029, 1979.
10. WOLF J, YESNER R, and PATNO ME. Evaluation of nitrogen mustard in prolonging life of patients with bronchogenic carcinoma. Cancer Chemother Rep 16:473-475, 1962.
11. UMSAWARDI T, BARKLEY HT, JR, BOOSER DG, ET AL. Role of prophylactic brain irradiation (PBI) during combined chemoradiotherapy (CCRT) for inoperable adeno and squamous cell lung cancer (ASCLC). Proc ASCO 1:148, 1982.
12. VOGL S, OHNUMA T, PERLOFF M, ET AL. Combination chemotherapy with adriamycin and cis-diamminedichloroplatinum in patients with neoplastic diseases. Cancer 38:21-26, 1976.
13. WOODMAN RS, SIRICA AE, GANG M, ET AL. The enhanced therapeutic effect of cis-platinum (II) diamminodichloride against L1210 leukemia when combined with cyclophosphamide or 1,2,-*bis* (3,4-dioxopiperazine-1-yl) propane or several other antitumor agents. Chemotherapy 18:169-183, 1973.
14. GRALLA RJ, CASPER ES, KELSEN DP, ET AL. Cis-platinum and vindisine combination chemotherapy for advanced carcinoma of the lung: a randomized trial investigating two dosage schedules. Ann Intern Med 95:414-520, 1981.

Adenocarcinoma of Unknown Primary Origin: Treatment With Vindesine and Doxorubicin[1,2]

John J. Fiore, David P. Kelsen,* Richard J. Gralla, Ephraim S. Casper, Gordon Magill, Edgar Cheng, and Manuel Ochoa, Jr[3]

Adenocarcinoma of unknown primary origin (ACUP) is a common oncologic problem for which there is no standard therapy. Forty-two patients with metastatic tumor were identified as having ACUP after extensive evaluation failed to reveal a primary site of disease. They were treated with an investigational chemotherapy regimen consisting of vindesine and doxorubicin. Of the 38 evaluable patients, six (16%) had major responses to chemotherapy. The median duration of response was 4 months. The median survival of the responding patients has not been reached, but is > 8 months. The median survival of the nonresponding patients was 6 months. Vindesine and doxorubicin were well tolerated. The major toxicity was leukopenia, with a median wbc count nadir of 2600/mm.^{3} We conclude that the combination of vindesine and doxorubicin has some activity in ACUP, but does not improve the response rate seen with other regimens. [Cancer Treat Rep 69:591–594, 1985]

Adenocarcinoma of unknown primary origin (ACUP) presents major diagnostic and therapeutic problems. Despite extensive clinical investigation, a primary site of disease is unlikely to be found; in approximately 15%–30% of patients, a primary site cannot be found even at autopsy (1,2). Therapy remains unsatisfactory. Since the malignancy is disseminated at the time of diagnosis, chemotherapy appears to be the most logical antineoplastic approach. However, most chemotherapy regimens yield response rates of < 20% and median survival times of < 4 months (3–7).

A rational approach to the treatment of such patients is to utilize chemotherapeutic regimens with agents which are active against the most likely primary site of disease, as well as those sites which are most responsive to chemotherapy. Of those patients with ACUP in whom a primary site is ultimately determined, cancer of the pancreas, lung, colon, and liver predominate. A smaller percent have gastric cancer, but < 3% have the more responsive tumors such as breast and ovarian cancer (1–3).

Vindesine (DVA) is a vinca alkaloid with demonstrated activity in non-small cell lung cancer and breast cancer (8,9). Doxorubicin is an anthracycline antibiotic with broad antitumor activity, including breast, pancreatic, and gastric cancer (10). This report describes a phase II trial designed to assess the response rate and toxicity of these two agents in combination in patients with ACUP.

MATERIALS AND METHODS

Forty-five patients with histologically proven adenocarcinoma, in whom the primary site of disease could not be located after thorough evaluation, were entered into this protocol from May 1980 to December 1982. All patients were required to have measurable or evaluable disease, a performance status of ⩾ 50%, and a signed statement of informed consent. The pretreatment evaluation to determine the primary site included a complete history and physical examination (with pelvic examinations in females), barium enema, upper gastrointestinal series, sigmoidoscopy, iv pyelogram, abdominal sonogram or computerized tomographic scan, chest x-ray, mammogram in females, and serum acid phosphatase in males. All patients had a cbc, biochemical screening profile, CEA, and ECG. Additional procedures to identify the primary site were performed at the discretion of the pri-

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[3] Solid Tumor Service (*J. J. Fiore, D. P. Kelsen, M. Ochoa, Jr, G. Magill,* and *E. Cheng*) and Developmental Chemotherapy Service (*R. J. Gralla* and *E. S. Casper*), Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY.

* *Reprint requests to:* David P. Kelsen, MD, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021.

mary physician.

Patients were treated with doxorubicin (20 mg/m^2 iv) weekly for 4 weeks and every 2 weeks thereafter; DVA (3 mg/m^2 iv) was also given weekly for 4 weeks and every 2 weeks thereafter. Treatment was continued until progression of disease was noted or unacceptable toxicity was encountered.

A cbc was measured on the day of each treatment. If the wbc count was 2000-3000 and/or the platelet count was 50,000-100,000/mm^3, drug doses were reduced by 50%. If either the wbc count was < 2000/mm^3 or the platelet count was < 50,000/mm^3, treatment was delayed for at least 1 week and until the blood cell counts had recovered.

Response criteria were as follows: *complete remission*—complete disappearance of all signs and symptoms of cancer for a minimum of 4 weeks; and *partial remission*—a ⩾ 50% reduction in the sum of the products of the perpendicular diameters of the measurable lesion(s) with no new or enlarging lesions for a minimum of 4 weeks. Patients with evaluable disease were considered improved if there was a definite decrease in the size of the evaluable lesion for a minimum of 4 weeks, with no increase in the size of other lesions or the appearance of new lesions. All such responses required confirmation by at least one other investigator. All response durations were measured from the beginning of chemotherapy until documented disease progression. Patients were considered adequately treated if they received two doses each of doxorubicin and DVA.

RESULTS

Forty-five patients were originally entered into the protocol. Three patients had major protocol violations and are excluded from further analysis. Patient characteristics and response data of the remaining 42 patients are presented in table 1. Major sites of disease at the time of diagnosis are listed in table 2. Only one patient had received prior chemotherapy.

Although a uniform diagnostic work-up was attempted, some patients did not have all the required investigations. The number of patients having each test performed is listed in table 3. Additional studies were frequently undertaken to further evaluate abnormal but nonspecific findings on initial x-rays or scans. These studies are presented in table 4.

Four patients received only one course of chemotherapy or did not return for follow-up evaluation; 38 patients are evaluable for response. One patient with measurable disease had a complete response, four with measurable disease had partial responses, and one with evaluable disease had marked improvement. Two of the responding patients relapsed and died primarily of uncontrolled central nervous system disease.

The primary site of disease was subsequently discovered ante mortem in two patients. The first patient was a 63-year-old white female with a negative work-up, including mammogram. Supraclavicular adenopathy completely disappeared for 12 months. She then developed erythema of her breast, and repeat mammography revealed a mass. A subsequent response has occurred with the combination of cyclophosphamide, methotrexate, 5-FU, vincristine, and prednisone. The second patient was a 63-year-old white female who was thought to have metastatic disease to the liver. When she failed to respond to chemotherapy, an exploratory laparotomy was performed. A primary cholangiocarcinoma of the liver was discovered, which required a hepatic trisegmentectomy for complete removal.

Toxicity.—Hematologic toxicity was moderate. Median wbc count nadir per patient was 2600/mm^3 (range, 900-6800), which usually occurred at Day 15. Dose attenuation was required at least once in 73% of the patients. Three patients had a wbc count nadir < 1000/mm^3 accompanied by fever which required hospitalization for iv antibiotics. Thrombocytopenia was mild; only two patients developed a platelet count < 100,000/mm^3 and

TABLE 1.—Patient characteristics*

Total	42
Evaluable	38
Age (yrs)	
Median	60
Range	32-74
Sex	
Male	18
Female	24
Performance status	
Median	70
Range	50-90
Major responders	6 (16%)†
Duration of response (mos)	
Median	4
Range	2-12
Median duration of survival (mos)	
Responders	8
Nonresponders	6

* Unless otherwise specified, values = No. of patients.

†One complete response, 4 partial responses, and 1 marked improvement.

TABLE 2.—Sites of disease at initial evaluation

Site	No. of patients
Lung	20
Lymph nodes	16
Bone	13
Liver	6
Intra-abdominal mass	5
Subcutaneous mass	5
Mediastinal mass	2
Testicle, breast, palate, brain, pericardium	1 each

TABLE 3.—Initial diagnostic evaluation

Diagnostic test	No. of patients/ No. evaluable (%)
Chest x-ray	42/42 (100)
Barium enema	38/42 (90)
Upper gastrointestinal series	34/42 (81)
Sigmoidoscopy or colonoscopy	15/42 (36)
Mammography*	23/24 (96)
Iv pyelogram	27/42 (64)
Abdominal sonogram or computerized tomographic scan	38/42 (90)
Serum acid phosphatase	10/18 (56)

*One female patient with a clinically suspicious breast nodule underwent biopsy without having mammography.

TABLE 4.—Special investigations

Diagnostic test	No. of patients/ No. evaluable (%)
Bronchoscopy	16/42 (38)
Upper gastrointestinal endoscopy	8/42 (19)
Breast biopsy	5/24 (21)
Chest computerized tomographic scan	4/42 (10)
Thyroid scan	5/42 (12)
ERCP	1/42 (2)

neither had clinical evidence of bleeding. The median platelet count nadir was 277,000/mm^3 (range, 37,000-665,000). Mild nausea and vomiting and alopecia were common. A peripheral neuropathy, secondary to DVA therapy, was seen in 11 patients, but reduction of DVA dosage was required in only three. One responding female received 610 mg/m^2 of doxorubicin. Left ventricular ejection fraction measured by radionuclide cineangiography was 37% with exercise, and the doxorubicin was discontinued. The patient subsequently died of her disease without clinical evidence of congestive heart failure.

Six patients had postmortem examinations. Three of these patients had adenocarcinoma of the lung, two had adenocarcinoma of the pancreas, and, in one patient, the primary site was not evident.

DISCUSSION

The general approach to patients with ACUP remains quite controversial, in part because the disease is a diagnosis of exclusion, is refractory to standard therapy, and has a variable prognosis. The low response rates seen in most chemotherapy studies are probably a reflection of the predominance of resistant tumors, such as pancreatic and lung cancers, in the clinical entity known as ACUP. The variable prognosis (in which some patients will die within a few weeks to months of diagnosis, while rare individuals will be alive for ⩾ 5 years) presumably represents some inherent biological property of the primary tumor, but does allow the option of expectant observation in occasional patients (11).

The two opposing views in this controversy can be summarized as follows. The first view is that ACUP is not a real entity, and that the clinical patterns of these patients are too diverse to allow any generalizations regarding treatment. The second view is that most medical oncologists frequently see patients with a metastatic adenocarcinoma in which the primary is not apparent, that the conventional diagnostic evaluation is fairly well established, and, finally, that treatment is needed for the vast majority of these symptomatic patients (12). We have accepted the second approach in the belief that, until newer diagnostic tests (either radiologic or histologic) can eliminate the uncertainty of the primary site, ACUP is a clinical entity in which investigative studies are reasonable.

Previous reports have demonstrated the poor response rates seen in patients with ACUP (3-7,13,14). Our study demonstrates that the combination of DVA and doxorubicin has some activity in this disorder, but does not improve the response rate or survival noted by most investigators.

It is difficult to compare the results of therapeutic trials in patients with ACUP. Anderson et al (15) reported a 50% response rate to the combination of cyclophosphamide, doxorubicin, and vincristine. However, it should be noted that the median age of their patients was 36 years and that extensive clinical investigations were not performed in their patients. Likewise, Woods et al (7) noted a 36% major response rate to mitomycin and doxorubicin as part of a randomized trial in ACUP, but many of their patients had abbreviated diagnostic evaluation as well. It is possible, therefore, that different patient populations are being treated, which may explain differences in the response rates.

Our response rate of 16% is disappointing, but it is consistent with the results of most other investigators. It is unlikely that substantial therapeutic progress will be made until better treatments are available for the underlying carcinomas which comprise this difficult clinical entity.

REFERENCES

1. NYSTROM JS, WEINER JM, ET AL. Metastatic and histologic presentations in unknown primary cancer. Semin Oncol 4:53-58, 1977.
2. DIDOLKAR MS, FANOUS N, ET AL. Metastatic carcinomas from occult primary tumors. Ann Surg 186:625-630, 1977.
3. MOERTEL CG, REITEMEIER RJ, ET AL. Treatment of the patient with adenocarcinoma of unknown origin. Cancer 30:1469-1472, 1972.
4. VALENTINE J, ROSENTHAL S, and ARSENEAU J. Combination chemotherapy for adenocarcinoma of unknown primary origin. Cancer Clin Trials 2:265-268, 1979.
5. SHILDT RA, KENNEDY PS, CHEN TT, ET AL. Management of patients with metastatic adenocarcinoma of unknown origin: a Southwest Oncology Group study. Cancer Treat Rep 67:77-79, 1983.

6. INDUPALLI SR, BEDIKIAN AY, and BODEY GP. Adenocarcinoma of unknown primary origin: impact of chemotherapy on survival. South Med J 74:1431-1435, 1981.
7. WOODS RL, FOX RM, ET AL. Metastatic adenocarcinomas of unknown primary site. N Engl J Med 303:87-89, 1980.
8. GRALLA RJ, RAPHAEL BG, GOLBEY RB, ET AL. Phase II evaluation of vindesine in patients with non-small cell carcinoma of the lung. Cancer Treat Rep 63:1343-1346, 1979.
9. SMITH IE, HEDLEY DW, POWLES TJ, ET AL. Vindesine: a phase II study in the treatment of breast carcinoma, malignant melanoma, and other tumors. Cancer Treat Rep 62:1427-1433, 1978.
10. PRATT W, and RUDDON R. The Anticancer Drugs. New York, Oxford University Press, 1979, pp 155-170.
11. STEWART JF, TATTERSALL MHN, ET AL. Unknown primary adenocarcinoma: incidence of overinvestigation and natural history. Br Med J 1:1530-1533, 1979.
12. NYSTROM JS, WEINER JM, ET AL. Identifying the primary site in metastatic cancer of unknown origin. JAMA 241:381-383, 1979.
13. JAJEDA J, LEGHA S, ET AL. Combination chemotherapy with 5-FU, adriamycin, cyclophosphamide and cis-platinum (FACP) in the treatment of adenocarcinoma of unknown primary (ACUP) and undifferentiated carcinomas (UCUP). Proc ASCO 2:237, 1983.
14. BEDIKIAN AY, BODEY G, ET AL. Sequential chemotherapy for adenocarcinoma of unknown primary. Am J Clin Oncol 6:219-224, 1983.
15. ANDERSON H, THATCHER N, RANKIN E, ET AL. VAC (vincristine, adriamycin, cyclophosphamide) chemotherapy for metastatic carcinoma from an unknown primary site. Eur J Clin Oncol 19:49-52, 1983.

Mathematical Model of Remission Duration in Acute Myelogenous Leukemia[1,2]

B. G. Birkhead[3,*]

A mathematical model of disease-free survival in acute myelogenous leukemia is formulated in terms of proliferation of the leukemic stem cell compartment. A survivorship function is described which allows for the possibility that some patients may be cured of their disease and which captures the main features of experimental remission-duration curves. The model is used to investigate the effect of the amount of residual disease and the growth rate of the stem cell population on the length of remissions and the chances for cure. A method of comparing clinical trials, with potential for explanatory inference, is exemplified. [Cancer Treat Rep 69:595–601, 1985]

In the treatment of acute myelogenous leukemia (AML) the achievement of remission with its accompanying survival advantage has long been the main clinical focus. The use of certain cytotoxic drugs intensively and in combination, however, has resulted in a reported remission rate > 50% (1-4). Consequently, emphasis has now shifted to the prevention of leukemic relapse or, equivalently, the prolongation of disease-free survival (DFS).

As far as DFS is concerned, the main treatment objectives consist of achieving a maximum reduction in both the size of the residual leukemic burden on completion of the induction and cytoreduction phases of treatment and in the rate of disease reprogression thereafter (5).

Historically, even with early single-agent chemotherapy regimens, there has been an identifiable (small) subgroup of patients enjoying extended survival in remission (6-10). The relative size of this group has increased markedly as a by-product of the intensification of early treatment (3) and a direct consequence of the use of bone marrow transplantation techniques (6,11). As follow-up periods extend, there is growing speculation that some of these patients are actually cured (7,10,12,13).

In this paper, a model derived first in the context of dose-response in infectious diseases is applied to DFS in AML through a description of leukemic cell population dynamics. It leads to a new survival function which has the required cure plateau and exhibits the general features of experimental remission-duration curves. It may, therefore, be useful in the analysis of clinical trials data. Through the model, the effects on remission duration of the amount of residual disease, its growth rate, and the rates of stem cell division and loss can be investigated. The significance of the proportion of malignant cells which are stem cells is also demonstrated.

MODEL

A stochastic model of disease proliferation has been assumed: a more realistic description than deterministic growth and one which allows for the possibility of residual disease extinction (cure). To minimize complexity we will restrict attention to the leukemic stem cell compartment; in accordance with the model of Mackillop et al (14), all other cells lack self-renewal capacity and will be at one of n transitional levels (representing different degrees of differentiation) in the cell division hierarchy or will be end cells. We assume a simple birth-death growth law. That is to say, all stem cells act independently in such a way that each has probabilities $b.\Delta t$ and $d.\Delta t$ of dividing into two stem cells (birth) or being lost to the population (differentiation/death) in any small time interval Δt. b and d are assumed time-independent constants, with $b > d$ (a "birth" advantage). It should be noted that these growth assumptions are somewhat artificial in that they smooth out the probability of division over time (unlike the cell cycle model), but for the growth of large numbers of cells (involving large numbers of divisions) the approximation becomes increasingly justified. For well-established malignant populations, growth under these rules becomes approximately exponential and the size of the stem cell compartment can be considered to be approximately proportional to the total

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[2] Supported by the Department of Health and Social Security and the Imperial Cancer Research Fund.

[3] Clinical Operational Research Unit, Department of Statistical Science, University College London, England.

[*] *Reprint requests to:* Dr. Brian G. Birkhead, Clinical Operational Research Unit, Department of Statistical Science, University College London, Gower St, London WC1E 6BT, England.

leukemic burden (14). For a population of "m" cells, by the random nature of the growth, there is a probability of eventual extinction of $(d/b)^m$.

The achievement of remission is idealized as the reduction of the leukemic stem cell population below some threshold size (N), which will be proportional to the total cell population threshold, which in turn will be fixed by the limits of detection. Relapse will occur when N is again exceeded. Suppose that at the end of remission induction/cytoreduction therapy, the residual (stem cell) leukemic burden has size x, which for a group of patients will be considered to be a random variable. For this initial formulation we will take a simple Poisson distribution of mean "r" to describe the variation of a fairly homogeneous group. Such a description may also be used for an individual patient whose residual burden is not known with certainty.

All of the above assumptions are represented diagrammatically in figure 1.

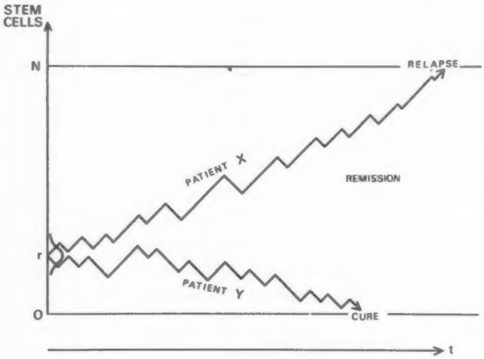

FIGURE 1.—Diagrammatic representation of the model.

EQUATIONS

Shortley (15) proposed an analogous model for the course of infectious diseases following a challenge dose of organisms. He developed earlier standard work of Saaty (16), who derived the approximate probability density function of the time t for a population to reach size N from a starting size n. Replacement of n by a Poisson variable of mean r (assumed small compared with N) leads by straightforward manipulation (15) to a modified density.

Here we take Shortley's model as applying to the population of leukemic *stem* cells. Mackillop's model of stem cell compartment growth, which is the basis of the assumed proportionality between established stem cell and total population sizes, is just a discrete analog of the growth process already described, formulated in terms of the stem cell renewal probability (the probability of a stem cell dividing to form two stem cells), which in this context is b/(b + d).

Here, t represents the time to leukemic relapse, and the equation of the DFS curve corresponding to Shortley's modified density function is given by equation 1

$$S(t) = 1 - \exp(-(q+y)) \sum_{k=1}^{\infty} \frac{q^k}{k!} \sum_{\ell=0}^{k-1} \frac{y^\ell}{\ell!} \qquad [1]$$

where $y = w.\exp(-pt)$; $p = b-d$; $q = r(1-d/b)$; and $w = N(1-d/b)$. S(t) is a family of curves depending on three parameters, p, q, and w. p is the net fractional growth rate of the disease (b−d) (17), and e^{-q} is the probability of cure (extinction of residual disease) (15). q/w is the ratio of the mean residual burden to the relapse threshold (r/N) and can be considered to represent the depth of the remission.

APPLICATIONS

(a) Example curves showing the dependence on the size of the (stem cell compartment) relapse threshold are shown in figure 2. For simplicity, and following examples in recent papers (18), b is taken equal to 1; for example, d = 0.99 and r = 100 cells. The effect of increasing N (or equivalently, of varying the proportionality constant between stem cell compartment size and total cell population size) is merely to translate the curves in the direction of increasing remission durations. The variance of relapse times is unaffected, as is the probability of cure.

Mackillop's approximate formula for the (steady-state) proportion of stem cells in the total malignant population is

$$Q = \left(\frac{2\alpha_s P - \beta}{2\alpha_s - \beta} \right) \cdot P^n \qquad [2]$$

where P is the probability per generation of stem cell renewal (= b/(b + d)), n is the number of transitional levels referred to earlier, and 1-β and 1-α_s are the probabilities per generation that cells may be lost from the end cell and stem cell populations, respectively.

In equation 2, if we assume for illustration that the *total* detectable leukemic burden at relapse is 10^9 cells, that $\beta = 0.9$, and that $\alpha_s = 1$, then for the parameter values used in figure 3, stem cell thresholds N of $10^4 \rightarrow 10^6$ (ie, $Q = 10^{-5} \rightarrow 10^{-3}$) would correspond to n values of between 11 and 14.

It should be remembered that the model from which equation 2 is derived is a minimal model which takes no account of such things as heterogeneity of cell cycle times or the effects of blood supply variations. Nonetheless, there is increasing support for the fundamental assumption of a proliferative hierarchy. Consistently low plating efficiencies observed in the culture data of human neoplastic cells and the clinical evidence of unexpected cures achieved with bulky tumors, using low linear energy transfer radiation, can be cited as support for most tumor

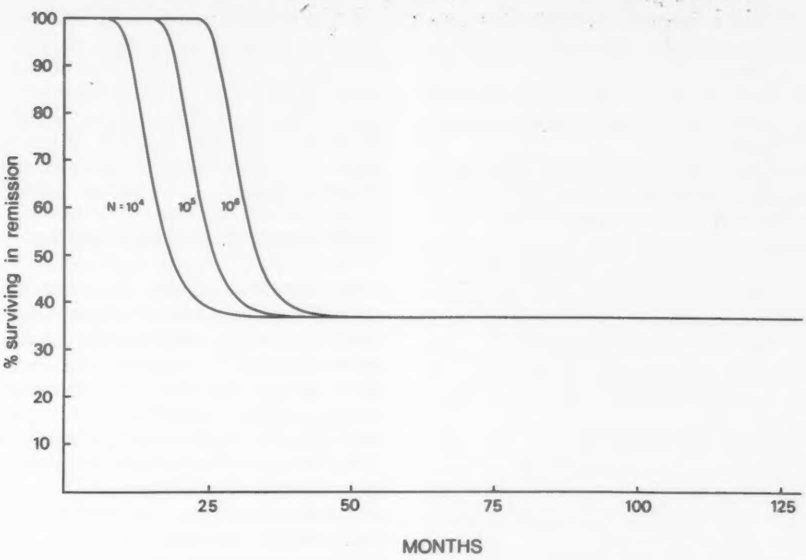

FIGURE 2.—Remission duration curves for different relapse thresholds ($b = 1$, $d = 0.99$, and $r = 10^2$).

cells lacking self-renewal capacity.

(b) Increasing residual disease levels (fig 3), while keeping all other parameters fixed, has the effect of diminishing the chances of cure and of decreasing both the median and variance of remission durations. Support, which of course, is model conditional, is thus given to the recent strategies which rely on the intensification of early therapy (19,20), and aims to secure as low as possible a value of r as early as possible.

(c) The role of maintenance therapy in AML has come into question in recent years (21,22). The main reasons for its failure to produce substantial benefits are the rate of selection and outgrowth of drug-resistant cell lines under repeated administration of the same drugs and cumu-

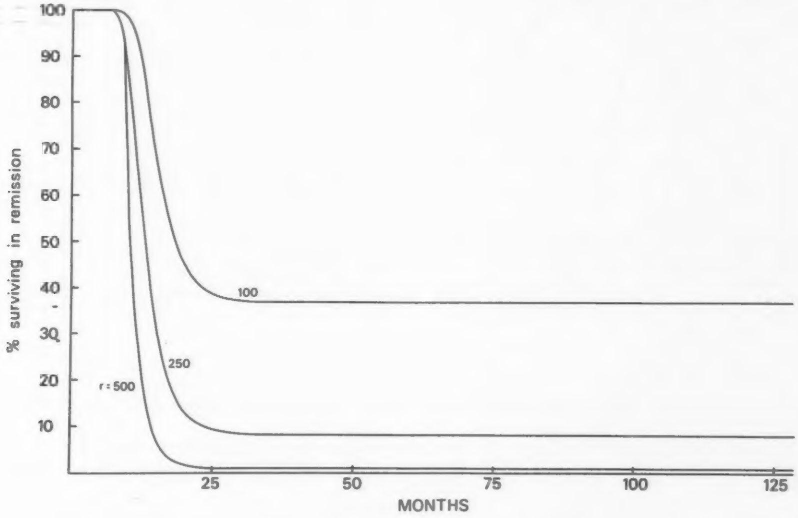

FIGURE 3.—Different levels of residual disease ($b = 1$, $d = 0.99$, and $N = 10^4$).

lative toxicity. Figure 4, therefore, probably only represents model-based expectations associated with effective maintenance and immunotherapy programs which succeed in slowing down the rate of disease reprogression. Over time, this should approximate increasing the rate, d, of loss of malignant stem cells.

(d) It might also be inferred from figure 4 that slower growing or highly differentiating tumors (higher d) would be expected to give rise to longer remission durations and increased cure probabilities, all other things being equal. But there is a compensating factor, since for malignant cell populations of the same size, the number of cells resistant to any chosen drug (the limiting size of residual disease) will itself depend on the value of d, and curability at least will to some extent be equalized. This can best be seen using the model of Goldie and Coldman (18).

Consider M leukemic stem cells at time t in the biological history of the disease, treated with "ideal" chemotherapy, so that *all* sensitive cells are instantaneously eradicated, leaving only the population of resistant cells as residual disease. Then (18), for b = 1,

$$E = \text{expected n}^{\circ}\text{. of resistant cells} \tag{3}$$

$$= \alpha \left[\left[t - \frac{d}{2(1-d)} \right] M + (t+\tfrac{1}{2}) \frac{d}{(1-d)} \exp(-(1-d)t) \right]$$

where α is the mutation rate towards resistance and

$$M(t) = \frac{1}{(1-d)} [\exp((1-d)t) - d.\exp(-(1-d)t)].$$

E clearly depends on the time for the stem cell compartment to reach size M, which in turn also depends on d.

If r is replaced in our model by E for the different values of d chosen in figure 4, then for $M = 10^6$ and $\alpha = 10^{-7}$, the corresponding values of t and r are given in table 1, resulting in the DFS parameter values shown in table 2. The corresponding curves are plotted in figure 5. While the remission duration advantage for higher values of d is maintained, the predisposition for increased probability of cure has been neutralized.

COMPARISONS OF TREATMENTS

For DFS curves arising from actual clinical trials it is possible to estimate the parameters of the model which "best" explain the data [so-called maximum likelihood estimation (23)]. Different treatments may then be compared through the respective parameter values themselves and their biological interpretation. This is exemplified in figure 6, which shows the Kaplan-Meier DFS plots of the two arms of a randomized controlled trial (24) set up to test the efficacy of immunotherapy as a means of maintaining remission. Although the numbers of patients involved are small, and little is known about the homogeneity of patient groups, the trial will adequately serve to demonstrate the methods of inference.

After the same induction and consolidation treatment, patients were randomized to receive either periodic courses of (specific + nonspecific) immunotherapy during remission (arm A) or no maintenance therapy at all (arm B). DFS has been measured from the end of 6 weeks of consolidation therapy. Table 3 shows the parameter estimates obtained when the model was fitted to each arm of the trial.

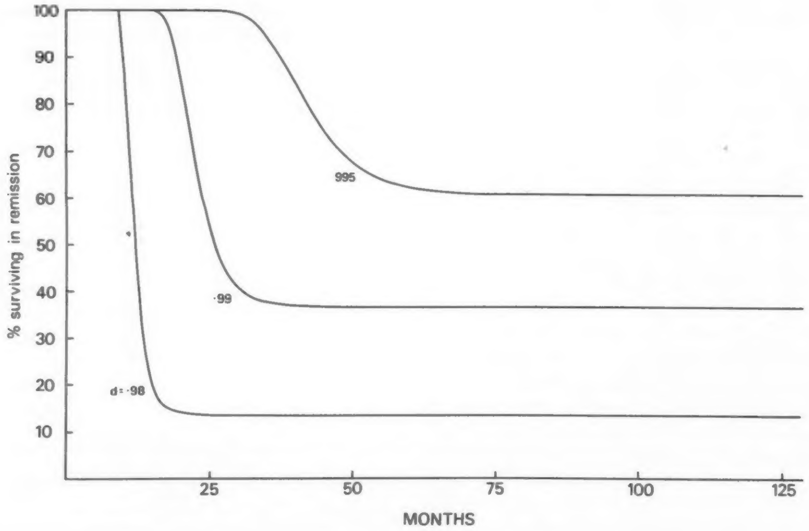

FIGURE 4.—Expected effect of maintenance strategies (increased d) ($b = 1$, $r = 10^2$, and $N = 10^5$).

Table 1

d	·98	·99	·995
t	495	922	1704
E=r	47	87	161

Table 2

d	·98	·99	·995
p	·02	·01	·005
q	·942	·873	·802
w	2000	1000	500

To exploit the structure of the model, the generalized likelihood ratio test (23) is used instead of the usual log-rank test, since it is then possible to perform tests for difference between individual parameters as well as for overall difference. In general, this means that when comparing two or more regimens, there is scope to extend investigation beyond the question of whether the curves are simply different, to consider in what ways (given the model) the treatments are different in their effects. For example: Does the maintenance treatment in X slow down the rate of disease reprogression compared to Y? (Compare estimates of the growth rate parameter p for the two trials.) Does Y produce significantly more cures than X? (Compare the q estimates, since e^{-q} gives the cure proportions.)

In this example the log-rank finding of an advantage in the immunotherapy arm was confirmed ($P < 0.01$).[4] In addition, however, a test on the p parameter showed that the superiority of A could be attributed in part to its effective retardation of the rate of disease reprogression ($P < 0.02$). There was, however, only limited support ($P < 0.20$) for an increased cure probability (lower q parameter). It should be noted that this latter comparison is sensitive to the small numbers of patients on the tail plateau and due caution is advised. Point estimates of the ratio r:N (q:s) for the two curves were almost coincident, as would be expected given the common induction and consolidation regimens used in both cases.

Of course, regimens in which any or all of the treatment phases are different (eg, different induction/maintenance) can be compared using this approach, and not just those, as in this example, where some are common. For *any* trial, estimates of p, q, and w can be made and may be compared with the corresponding estimates for any other trial.

CONCLUSION

This model of remission duration has been based on simple assumptions about the effects of treatment and the kinetics of AML.

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[4] *P* values are derived from the χ^2 approximation for the distribution of the likelihood ratio statistic.

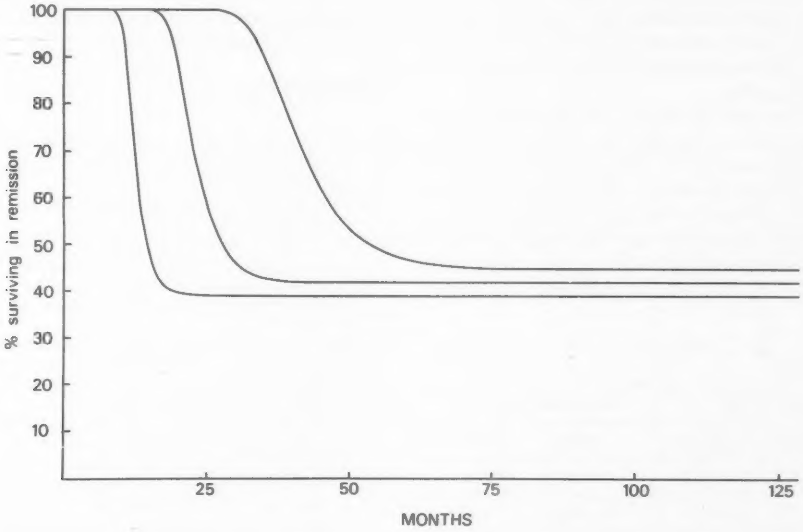

FIGURE 5.—Incorporation of the dependence of the resistant cell population size on the rate of stem cell differentiation or loss (b = 1 and $N = 10^5$).

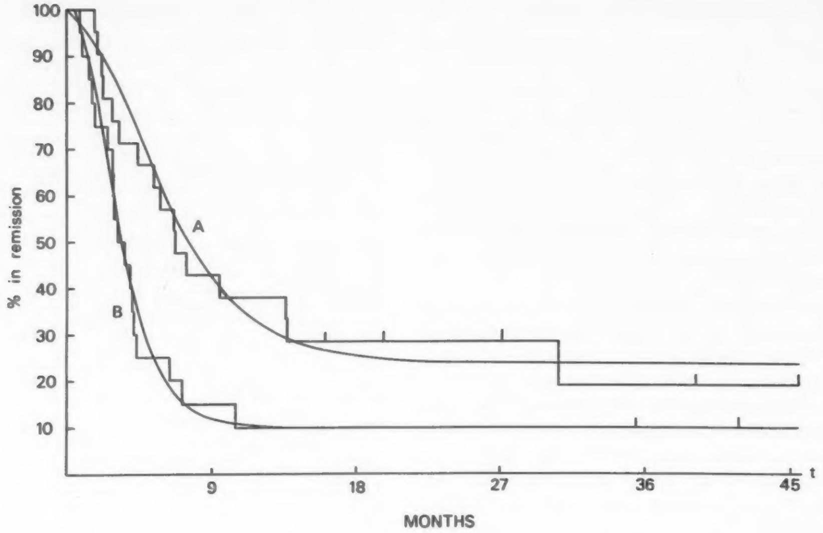

FIGURE 6.—Model fitted to the 2 arms of an immunotherapy trial.

It should be noted that the several biological assumptions on which the model rests may, at best, only approximate the real situation. In particular, the description of tumor growth adopted takes no explicit account of the cell cycle and, for well-established disease, restricts attention to near exponential growth (perhaps not too unrealistic during the subclinical phase). The effects of relaxing both of these constraints require further investigation. The notion of a proliferative hierarchy of cells and, in particular, of a stem cell subpopulation is central to the model, and more direct evidence of how realistic this is, is still required.

The model has led to an alternative survivorship function which may prove to be useful in clinical trials analysis, since it possesses the key features of many experimental curves. Given the model, different remission prolongation strategies can be simulated to predict their expected effects on chances for cure and times to relapse. For homogeneous groups of patients, data from actual clinical trials may be fitted to estimate the model's parameters and to provide a method of inference based on the assumed structure. Future work will include incorporating more patient-to-patient variation for each of the existing parameters, eg, using distributions other than Poisson for residual disease.

Table 3

	Immunotherapy		No Maintenance	
p	·0093	(·0024)	·0173	(·0032)
q	1·4515	(·3720)	2·3228	(·6010)
w	6·9981	(2·410)	10·6562	(3·421)

Figures in parentheses represent estimates of the standard deviations.

It is hoped that models such as the one described here, produced by formalizing plausible biological assumptions in relation to different treatment strategies, will prove to be useful to medical and scientific investigators, both for treatment design and evaluation.

REFERENCES

1. GLUCKSBERG H, CHEEVER MA, FAREWELL VT, ET AL. High dose combination chemotherapy for acute non-lymphocytic leukemia in adults. Cancer 48:1078-1081, 1981.
2. GALE RP, and CLINE MJ. High remission induction rate in acute myeloid leukaemia. Lancet 1:497-499, 1977.
3. BELL R, ROHATINER AZ, SLEVIN ML, ET AL. Short-term treatment of acute myelogenous leukaemia. Br Med J 284:1221-1224, 1982.
4. REES JKH, SANDLER RM, CHALLENDER J, ET AL. Treatment of acute myeloid leukaemia with a triple cytotoxic regimen. Br J Cancer 36:770-774, 1977.
5. CROWTHER D. A rational approach to the chemotherapy of human malignant disease. I. Br Med J 274:216-218, 1974.
6. POWLES RL, PATU G, and RAGHAVAN D. Curability of acute leukaemia. Top Rev Haematol 1:186-219, 1980.
7. WHITTAKER JA. Advances in the management of adult myelogenous leukaemia. Br Med J 281:960-964, 1980.
8. FREIREICH EJ. The criteria for curability of adult acute leukaemia. Leukaemia Research Fund 15th Annual Guest Lecture, 1980.
9. JACQUILLAT C, WEIL M, GEMON MF, ET AL. Evaluation of 216 four-year survivors of acute leukemia. Cancer 32:286-293, 1973.
10. PETERSON BA, and BLOOMFIELD CD. Long-term disease-free survival in acute non-lymphocytic leukaemia. Blood 57:182-185, 1981.
11. THOMAS ED, and BRUCKNER CD. Marrow transplantation for acute

non-lymphoblastic leukemia in first remission. N Engl J Med 301:597, 1980.

12. WHITTAKER JA. Long survival in acute myelogenous leukaemia: an international collaborative study. Br Med J 282:692-695, 1981.
13. SPIERS ASD, GOLDMAN JM, CATOVSKY D, ET AL. Prolonged remission maintenance in acute myeloid leukaemia. Br Med J 2:544-547, 1977.
14. MACKILLOP WJ, CIAMPI A, TILL JE, ET AL. A stem cell model of human tumor growth: implications for tumor cell clonogenic assays. JNCI 70:9-16, 1983.
15. SHORTLEY G. A stochastic model for distributions of biological response times. Biometrics 21:562-583, 1965.
16. SAATY T. Elements of Queueing Theory. London, McGraw-Hill, 1981.
17. RUBINOW SI, and LEBOWITZ JL. A mathematical model of the acute myeloblastic leukemic state in man. Biophys J 16:897-909, 1976.
18. GOLDIE JH, and COLDMAN AJ. Quantitative model for multiple levels of drug resistance in clinical tumors. Cancer Treat Rep 67:923-931, 1983.
19. PREISLER H, BROWMAN G, HENDERSON E, ET AL. Treatment of acute myelocytic leukaemia: effects of early intensive consolidation. Proc Am Assoc Cancer Res and ASCO 21:443, 1980.
20. OMURA GA, VOGLER WR, and LYNN MJ. A controlled clinical trial of chemotherapy versus B.C.G. immunotherapy versus no further therapy in A.M.L. Proc Am Assoc Cancer Res and ASCO 18:272, 1977.
21. LISTER TA, WHITEHOUSE JMA, OLIVER RTD, ET AL. Chemotherapy and immunotherapy for acute myelogenous leukemia. Cancer 46:2142-2148, 1980.
22. POWLES RL, SELBY PJ, PALU G, ET AL. The nature of remission in acute myeloblastic leukaemia. Lancet 2:674-676, 1979.
23. KALBFLEISCH JD, and PRENTICE RD. The Statistical Analysis of Failure Time Data. New York, J. Wiley and Sons, 1980.
24. ZUHRIE SR, HARRIS R, FREEMAN CB, ET AL. Immunotherapy alone versus no maintenance treatment in acute myelogenous leukaemia. Br J Cancer 41:372, 1980.

High-Dose Etoposide in the Treatment of Relapsed Primary Brain Tumors[1]

G. P. Finn,[2] T. Bozek,[2] R. L. Souhami,[2,*] M. L. Slevin,[3] and D. G. T. Thomas[4]

Twenty-four patients with relapsed primary intracranial tumors were treated with high-dose etoposide (600–1000 mg/m^2). All patients had received previous radiotherapy and 15 had had previous chemotherapy. The treatment was well-tolerated, with little toxicity. Only one patient showed a partial response, which was maintained for 78 weeks. Four patients had stable disease, for a median of 15 weeks. Etoposide failed to show useful activity in relapsed brain tumors. [Cancer Treat Rep 69:603–605, 1985]

Primary malignant intracranial tumors continue to have a poor prognosis following surgery and radiotherapy. The number of chemotherapeutic agents with activity is limited. Responses are seen with vincristine (1), nitrosoureas (2), procarbazine (3), and cisplatin (4), but additional active agents are needed. Etoposide and teniposide are semisynthetic analogs of podophyllotoxin, a plant derivative. Their chemical characteristics of low molecular weight and high lipid solubility make them suitable candidates for possible use in brain tumors. Both agents have been used extensively in a wide range of tumors and show a similar range of activity. Teniposide has been used in relapsed primary brain tumors, mainly gliomas, with reported response rates of 20%–35% (5–8).

There is little information on the use of etoposide in primary brain tumors. We have therefore treated 24 patients who have relapsed on primary treatment. We have used a very high-dose schedule, since previous reports have indicated that this is safe and may increase the response rate of the tumor (9).

PATIENTS AND METHODS

Twenty-four patients were studied. Patient characteristics are shown in table 1. All patients had received previous radiotherapy, with tumor doses from 45.5 to 62.5 Gy. Fifteen patients had received previous chemotherapy as part of an adjuvant program for malignant gliomas. The agents used were procarbazine (100 mg/m^2, Days 1-10), lomustine (50 mg/m^2, Day 1), and vincristine (1.4 mg/m^2, Day 1), repeated at 6-week intervals. One patient received a single course of doxorubicin on relapse from procarbazine, lomustine, and vincristine.

All patients had progressive disease as shown by clinical deterioration and/or an expanding lesion on computerized tomographic (CT) brain scan. The histological diagnosis had been confirmed in all patients but one (table 1). Patients had normal blood cell counts and renal and hepatic functions at outset. Steroid therapy was maintained at the same dose or reduced during chemotherapy.

Treatment with etoposide was given by infusion in normal saline at a rate of 1 mg/minute. The total dose was administered in two separate infusions of equal quantity on each of 2 consecutive days. Four patients received a total dose of 600 mg/m^2; the other patients received 1 g/m^2. Treatment was repeated at 3-week intervals. On

TABLE 1.—Patient characteristics

Characteristic	No. of patients
Mean age in yrs (range): 46 (2–71)	
Male/female	16/8
Diagnosis	
Glioma	
Grade III–IV	15
Grade I–II	4
Medulloblastoma	2
Pinealoblastoma	1
Malignant choroid plexus papilloma	1
Unknown histology	1
Previous chemotherapy	15
Previous radiotherapy	24

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[2] Department of Radiotherapy and Oncology, University College Hospital, London, England.

[3] Department of Medical Oncology, St. Bartholomew's and Hackney Hospitals, London, England.

[4] Gough-Cooper Department of Neurological Surgery, Institute of Neurology, London, England.

[*] *Reprint requests to:* Dr. R. L. Souhami, Department of Radiotherapy and Oncology, University College Hospital, Gower St, London WC1E 6JJ, England.

each visit the patients underwent full clinical examination, blood cell count, and biochemical test of liver and renal functions. CT brain scan was performed at 9-week intervals unless otherwise indicated.

Complete response was defined as the disappearance of the lesion on CT scan and improvement in neurological symptoms and signs. Partial response was defined as a > 50% reduction in maximum tumor diameter on CT scan and clinical improvement. Stable disease was defined as failure to deteriorate or clinical improvement with no change in CT scan.

Blood samples were taken at frequent intervals during and after the etoposide infusions in three patients on 2 consecutive days. The samples were centrifuged and plasma was separated and stored at -20°C until assay. In one patient a CSF sample was obtained by lumbar puncture during the plateau phase of the infusion on Day 2. Plasma and CSF etoposide concentrations were measured using reverse-phase high-performance liquid chromatography (10).

RESULTS

All patients received at least one course of treatment and were considered evaluable. In nine patients there was rapid progression of disease after the first cycle of chemotherapy. The remaining 15 patients received between two and 13 courses.

Response to treatment is summarized in table 2. Of 24 patients, 19 showed disease progression between one and four courses. Four patients had stable disease maintained for 12–24 weeks (median, 15). Only one patient showed a partial response, with improved neurological symptoms and signs and a smaller lesion on CT scan. This responding patient had an initial diagnosis of grade I–II astrocytoma which relapsed 3 years later as a grade II–III lesion. She had received no previous chemotherapy. Treatment was given over 46 weeks and was then discontinued because of stable clinical signs and CT scan. She remained off treatment for a further 32 weeks when she began to deteriorate, although CT scan was unchanged.

Toxicity

The infusion was well-tolerated, with little nausea or vomiting. The major toxic effect was hematological, with four cycles being delayed because of neutropenia (< 2 × 10^9 cells/L) and five because of thrombocytopenia (< 100 × 10^9 cells/L). Only one patient had symptomatic neutropenia, with septicemia requiring iv antibiotics. Mild transient abnormalities in liver function tests were observed in several patients. These abnormalities persisted in one patient but the liver was normal at postmortem examination. Other toxic effects included a transient erythematous rash, a blood culture-negative pyrexia, and a single episode of hiccoughs.

Pharmacology

Plasma concentrations of etoposide in the three patients on Days 1 and 2 are shown in figure 1. Plateau concentrations (end infusion levels) were 12.3, 21.2, and 34.5 μg/ml on Day 1 and 12.7, 27.4, and 35.3 μg/ml on Day 2 in the three patients. Similarly, the areas under the curve for the three patients were 282, 375, and 602 μg/ml · hours on Day 1 and 314, 378, and 637 μg/ml · hours on Day 2. The single CSF concentration of etoposide was 0.57 μg/ml, 3.9% of the simultaneous plasma concentration.

DISCUSSION

This investigation has shown that etoposide does not have useful activity in relapsing brain tumors. One patient achieved a partial response, which lasted 78 weeks. These results are in contrast to data using teniposide in which response rates of up to 30% are reported (5–8). These studies have usually recorded clinical

TABLE 2.—Treatment given with best response, duration, and diagnosis

No. of patients	No. of courses of treatment	Response*	Relapse-free period (wks)	Diagnosis
9	1	NR		
5	2	NR		
4	3	3 NR		
		1 SD	12	Grade II astrocytoma
3	4	2 NR		
		1 SD	12	Grade II astrocytoma
1	6	SD	18	Grade II astrocytoma
1	8	SD	24	Medulloblastoma
1	13	PR	78	Grade II–III astrocytoma

* Values = No. of patients. NR = no response; SD = stable disease; and PR = partial response.

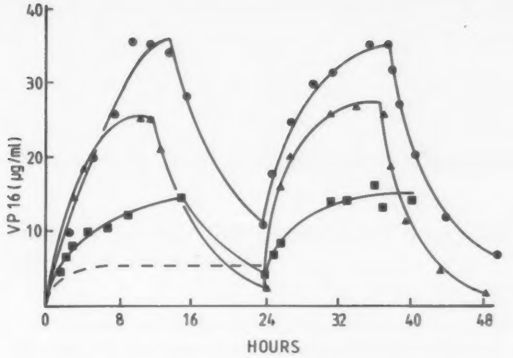

FIGURE 1.—Serum etoposide levels during and after the period of infusion in 3 patients. Total dose, 1 g/m^2 (each infusion, 500 mg/m^2). Dashed lines are values recorded by D'Incalci et al (ref 11) for infusion of 100 mg/m^2 over 24 hrs.

improvement alone and the response to teniposide would probably be considerably lower if CT scan criteria had been included. Lack of disease progression has not been included as evidence of response in our study, since the interpretation of stable disease is open to question.

We used etoposide at a high dose to increase the likelihood of showing activity. The serum concentrations of etoposide were significantly higher than those found with conventional-dose infusions of 100 mg/m^2 for 24 hours, where plateau levels of approximately 5 μg/ml have been reported (11). The wide between-patient variability in plasma etoposide concentrations has previously been described by several investigators (11). The ratio of CSF to plasma concentration of etoposide in the one patient reported here is similar to that previously published (12). It has been suggested that etoposide concentrations in the tumor may be much higher than those in the CSF (13).

We have therefore failed to show useful activity of etoposide in primary brain tumors. Although more than one-half of our patients were pretreated, the high-dose schedule employed makes it probable that high activity would have been detected. It is probable that etoposide will not be a useful agent in primary brain tumors.

REFERENCES

1. SMART CR, OTTOMAN RE, ROCHLIN DB, ET AL. Clinical experience with vincristine (NSC-67574) in tumors of the central nervous system and other malignant diseases. Cancer Chemother Rep 52:733–741, 1968.
2. ROSENBLUM ML, REYNOLDS AF, JR, SMITH KA, ET AL. Chloroethylcyclohexyl-nitrosourea (CCNU) in the treatment of malignant brain tumors. J Neurosurg 39:306–314, 1973.
3. KUMAR ARV, RENAUDIN J, WILSON CB, ET AL. Procarbazine hydrochloride in the treatment of brain tumour: phase II study. J Neurosurg 40:365–371, 1974.
4. KHAN AB, D'SOUZA BJ, WHARAM MD, ET AL. Cisplatin in recurrent childhood brain tumors. Cancer Treat Rep 66:2013–2020, 1982.
5. GEROSA MA, DI STEFANO E, OLIVI A, ET AL. VM-26 monochemotherapy trial in the treatment of recurrent supratentorial gliomas: preliminary report. Surg Neurol 15:128–134, 1981.
6. KESSINGER A, LEMON HM, and FOLEY JF. VM-26 as a second drug in the treatment of brain tumors. Cancer Treat Rep 63:511–512, 1979.
7. SKLANSKY BD, MANN-KAPLAN RS, REYNOLDS AF, ET AL. 4'Demethylepipodophillotoxin-thenylidene-glucoside (PTG) in the treatment of malignant intracranial neoplasms. Cancer 33:460–467, 1974.
8. SPREMULLI E, SCHULZ JJ, SPECKHART VJ, ET AL. Phase II study of VM-26 in adult malignancies. Cancer Treat Rep 64:147–149, 1980.
9. WOLFF SN, FER MF, MCKAY CM, ET AL. High dose VP16-213 and autologous bone marrow transplantation for refractory malignancies: a phase I study. J Clin Oncol 11:701–705, 1983.
10. HARVEY VJ, JOEL SP, JOHNSTON A, ET AL. High-performance liquid chromatography of etoposide (VP16-213) in plasma and urine. J. Chromatogr, 1984. In press.
11. D'INCALCI M, FARINA P, SESSA C, ET AL. Pharmacokinetics of VP16-213 given by different administration methods. Cancer Chemother Pharmacol 7:141–145, 1982.
12. CREAVEN PJ. The clinical pharmacology of VM-26 and VP16-213. A brief overview. Cancer Chemother Pharmacol 7:133–140, 1982.
13. STEWART DJ, RICHARD M, HUGENHOLTZ H, ET AL. VP16 and VM26 penetration into human brain tumours. Proc Am Assoc Cancer Res 24:133, 1983.

Phase I Study of Divided-Dose Vinblastine in Advanced Malignancy[1]

James A. Young,* Debbie Westlake, George W. Schnetzer, III, Alan M. Keller, John M. Sexauer, and Lee N. Newcomer[2]

Thirty-one patients with advanced malignancy received vinblastine as an iv bolus at 0 and 48 hours of a 21-day cycle. Divided-dose vinblastine may be given with acceptable toxicity in heavily pretreated patients; leukopenia and neuropathy were the dose-limiting toxic effects. One patient with breast cancer and one with ovarian cancer responded. Serum vinblastine levels with this program approximate those achieved by a 5-day continuous infusion of vinblastine in standard dose. [Cancer Treat Rep 69:607–610, 1985]

Tumor cell kill may be enhanced for cell cycle-specific drugs by modification of the dose schedule to result in an increased drug concentration-time of exposure product. Investigators have begun clinical trials of prolonged infusion of vinblastine and other vinca alkaloids, with the goal of increasing antineoplastic effect (1–3). Vinblastine infusion was initially reported to yield a high response in patients with refractory breast cancer, including those previously exposed to vinblastine bolus, but subsequent investigators have been unable to duplicate this experience (3,4). Schulman et al (5) completed a phase I clinical trial of vinblastine given in four bolus doses over 48 hours and observed responses in patients with refractory lung and ovarian cancers.

Pharmacokinetic studies of bolus vinblastine injection have demonstrated a triphasic serum decay curve, with a prolonged terminal half-life of 20 hours (6,7). Patients receiving a bolus injection of 10 mg/m^2 are observed to have detectable serum vinblastine levels of 1–2 ng/ml at 48 hours after dosing (7). In a phase I trial of 5-day vinblastine infusion, patients treated with the highest tolerated dose of 1.75 mg/m^2/24 hours achieved a mean plateau serum vinblastine level averaging 2 ng/ml at 24 hours and maintained this level for the duration of the infusion (8). The pharmacokinetic data suggest that divided-dose bolus injections of vinblastine, spaced at 48-hour intervals, might closely approximate serum drug levels achieved by a continuous-infusion schedule.

MATERIALS AND METHODS

Patient eligibility.—Patients with histologically documented advanced malignancy who had exhausted all standard therapy were accepted for study. Patients were required to provide informed consent; all had an Eastern Cooperative Oncology Group performance status of 3 or better and an expected survival of at least 2 months. Adequate bone marrow, hepatic, and renal functions were required, as defined by the following laboratory criteria: wbc count $> 3500/mm^3$, platelet count $> 100{,}000/mm^3$, serum creatinine < 1.6 mg/dl, serum bilirubin < 1.6 mg/dl, and serum SGOT less than twice normal.

Drug therapy.—Vinblastine (Eli Lilly and Co) was given as an iv bolus over 5 minutes via a peripheral vein. Patients were treated at starting doses of 3.0, 4.5, or 6.0 mg/m^2 at 0 and 48 hours of a 21-day cycle. Individual patients were eligible for a 25% dose escalation if, on Day 15 of the previous cycle, the wbc count was $> 2500/mm^3$ and the platelet count was $> 100{,}000/mm^3$.

Vinblastine serum levels.—Vinblastine radioimmunoassay was performed as described previously using commercially available radiolabeled vinblastine (^{3}H-vinblastine sulfate; Amersham) and rabbit vinblastine antiserum (provided by Dr. M. A. Root, Lilly Research Laboratory) (9). Serum samples were obtained 5 minutes after the 0-hour injection, immediately prior to the 48-hour injection, and at 96 hours. Samples were allowed to clot, were centrifuged, and were frozen for storage at -70°C.

RESULTS

Patient Outcome

Thirty-one patients received a mean of 2.7 cycles

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[2] Natalie Warren Bryant Cancer Center, St. Francis Hospital, and Warren Research Institute, Tulsa, OK.

* *Reprint requests to:* James A. Young, MD, Natalie Warren Bryant Cancer Center, St. Francis Hospital, 6161 S Yale Ave, Tulsa, OK 74136.

(range, one to ten) of divided-dose vinblastine. One patient died of progressive metastatic malignant melanoma 2 weeks after starting treatment. One patient with metastatic ovarian cancer received only half of her planned first cycle and died 2 weeks later of metabolic encephalopathy and sepsis unrelated to treatment. Characteristics of the remaining 29 patients evaluable for toxicity and pharmacokinetic data are shown in table 1.

TABLE 1.—Patient characteristics

Characteristic	No. of patients
Total evaluable	29
Median age in yrs (range): 58 (31-82)	
Sex (male/female)	3/26
Mean No. of cycles (range): 2.7 (1-10)	
Primary tumor types	
Breast adenocarcinoma	15
Ovarian adenocarcinoma	9
Renal cell carcinoma	2
Other	3
Prior therapy	
Combination chemotherapy	29
Radiation therapy	4
Performance status	
0-1	8
2-3	21
Starting dose (mg/m^2)	
3.0	15
4.5	8
6.0	6

Toxicity

Five patients (one of 15 at the 3.0-mg/m^2 dose level, two of eight at the 4.5-mg/m^2 dose level, and two of six at the 6.0-mg/m^2 dose level) required hospitalization for neutropenia and fever, but there were no treatment-related deaths. Of 15 patients treated at the 3.0-mg/m^2 dose level, only one experienced a wbc count $< 2000/\text{mm}^3$. Significant thrombocytopenia (platelet count $< 50{,}000/\text{mm}^3$) was not observed at any dose. Table 2 shows mean nadir wbc and platelet counts.

Five patients (one of 15 at the 3.0-mg/m^2 dose level, one of eight at the 4.5-mg/m^2 dose level, and three of six at the 6.0-mg/m^2 dose level) experienced severe transient ileus or constipation temporally related to treatment, and nasogastric suction was required in three patients. In all patients, the ileus resolved within 5 days. One patient developed superficial phlebitis of a peripheral vein. No other significant toxic effects occurred.

Response

One patient with metastatic ovarian cancer, recurrent after combination chemotherapy with cisplatin and hexamethylmelamine, had a surgically documented complete response after six cycles of therapy and is clinically disease-free 12 months after beginning therapy. One patient with metastatic breast cancer, recurrent after combination chemotherapy and hormonal therapy, had a complete response of 2 months' duration after a single cycle, but refused further therapy because of toxicity.

Pharmacokinetics

Table 2 shows serum vinblastine levels for the three doses studied. Adequate data were obtained on six patients at each dose level. The patient-to-patient variability in the drug levels achieved by a given dose is reflected in the standard deviations. Cycle-to-cycle variation in drug levels for a given patient and dose was modest. Figure 1 shows a comparison of pharmacokinetic data from a previous study for a 5-day continuous infusion of vinblastine (1.75 mg/m^2/24 hours), with data from the present study for the divided-dose schema with 4.5 mg/m^2 bolus at 0 and 48 hours.

DISCUSSION

This phase I study was designed to provide a clinical and pharmacokinetic basis for the use of divided-dose vinblastine. The observed pattern of toxicity for the program was similar to that reported for vinblastine infusion. Neuropathy and neutropenia were dose-limiting toxic effects, and thrombocytopenia was modest. Day-15 myelosuppression (table 2) was more severe at a dose of 4.5 mg/m^2 than at a dose of 6.0 mg/m^2; this probably reflects a difference in patient characteristics of the relatively small number of patients treated at those doses. In our group of patients who were heavily pretreated and had relatively poor performance status, doses of 3.0-6.0

TABLE 2.—Myelosuppression and serum drug levels

	Day-15 myelosuppression (mean ± SD)		Vinblastine drug levels (mean ± SD)*		
Dose (mg/m^2)	Wbc count (× 10^3/mm^3)	Platelet count (× 10^3/mm^3)	5 mins	48 hrs (trough)	96 hrs
3.0	4.4 ± 2.7	263 ± 210	97 ± 35	1.2 ± 0.4	1.3 ± 0.6
4.5	1.6 ± 1.0	110 ± 54	74 ± 6	1.6 ± 0.5	2.9 ± 1.0
6.0	2.8 ± 2.1	186 ± 136	116 ± 57	2.9 ± 1.1	3.3 ± 1.3

* Values (ng/ml) = mean ± SD for 6 patients at each dose level.

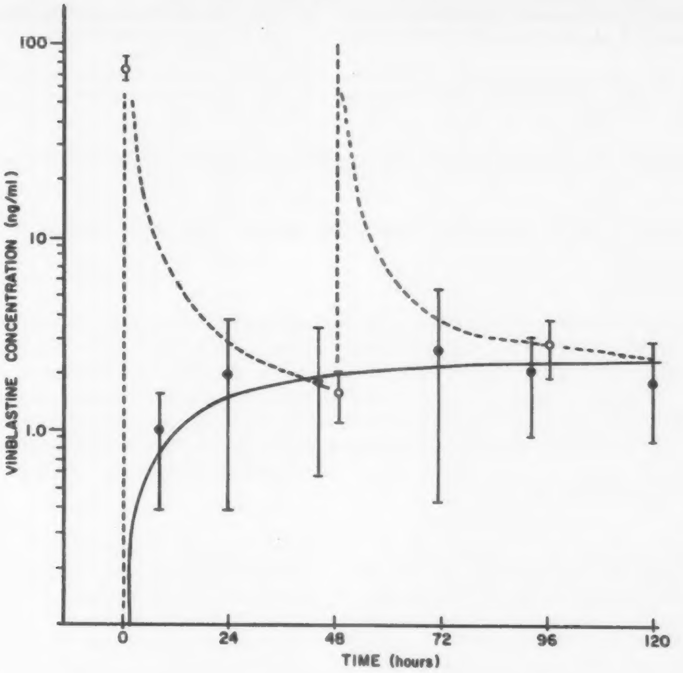

FIGURE 1.—Comparison of divided-dose (----) and continuous-infusion (——) pharmacokinetics. Continuous-infusion curve is based on patients treated with vinblastine (1.75 mg/m^2/24 hrs) in a previous study (8). Mean drug levels and SDs are shown with solid dots. Divided-dose curve is based on estimates of serum vinblastine half-life from the literature (6,7), coupled with measured peak, 48-hr trough, and 96-hr levels (open dots) from the current study at a dose of 4.5 mg/m^2 at 0 and 48 hrs.

mg/m^2 at 0 and 48 hours of a 21-day cycle were relatively well-tolerated and safe, with no treatment-related deaths observed. In future phase II trials in patients with extensive prior therapy, we will begin treatment at 4.0 mg/m^2 at 0 and 48 hours and escalate the dose on subsequent cycles based on nadir counts.

In our prior phase I study of 5-day vinblastine infusion, the maximum safely tolerated dose was 1.75 mg/m^2/24 hours (8). Patients were found to achieve maximum serum vinblastine levels at about 24 hours and that level was maintained for the duration of the infusion; there was wide patient-to-patient variability in the plateau level achieved. At the 1.75-mg/m^2/24 hour dose, the mean 96-hour vinblastine level was 2.5 ± 1.36 ng/ml (8). In the present study, the mean 96-hour vinblastine level was 2.9 ± 1.0 ng/ml for the 4.5-mg/m^2 dose. Thus, trough serum vinblastine levels for the divided-dose regimen may approximate the plateau level achieved by the 5-day infusion program (fig 1); the drug concentration-time of exposure product for the two schedules may not differ significantly.

Because of the vesicant properties of the drug, vinblastine continuous infusion usually requires a central venous catheter. Outpatient infusion requires careful supervision and expensive pump devices, and many patients require hospitalization for therapy. The divided-dose schema provided an inexpensive, convenient, and well-tolerated alternative to continuous vinblastine infusion. Phase II trials of divided-dose vinblastine are planned in breast and ovary cancers.

REFERENCES

1. BODEY GP, YAP H-Y, YAP B-S, ET AL. Continuous infusion vindesine in solid tumors. Cancer Treat Rev 7:39-45, 1980.
2. JACKSON DV, SETHI VS, SPURR CL, ET AL. Intravenous vincristine infusion: phase I trial. Cancer 48:2559, 1981.
3. YAP H-Y, BLUMENSCHEIN GR, KEATING MJ, ET AL. Vinblastine given as a continuous 5-day infusion in the treatment of refractory advanced breast cancer. Cancer Treat Rep 64:279-283, 1980.
4. TANNOCK I, ERLICHMAN C, PERRAULT D, ET AL. Failure of 5-day vinblastine infusion in the treatment of patients with advanced refractory breast cancer. Cancer Treat Rep 66:1783-1784, 1982.
5. SCHULMAN P, BUDMAN DR, VINCIGUERRA V, ET AL. Phase I evaluation of divided dose vinblastine sulfate. Cancer Chemother Pharmacol 5:67-69, 1980.

6. OWELLEN RJ, HARTKE CA, and HAINS FO. Pharmacokinetics and metabolism of vinblastine in humans. Cancer Res. 37:2597-2602, 1977.
7. NELSON RL, DYKE RW, and ROOT MA. Comparative pharmacokinetics of vindesine, vincristine, and vinblastine in patients with cancer. Cancer Treat Rev 7:17-24, 1980.
8. YOUNG JA, HOWELL S, and GREEN MR. Pharmacokinetics and toxicity of 5-day continuous infusion of vinblastine. Cancer Chemother Pharmacol 12:43, 1984.
9. SETHI VS, BURTON SS, and JACKSON DV. A sensitive radioimmunoassay for vincristine and vinblastine. Cancer Chemother Pharmacol 4:183-187, 1980.

Phase I-II Clinical Trial of Thymidine, 5-FU, and PALA Given in Combination[1,2]

Delia F. Chiuten,[3,*] Manuel Valdivieso,[3,4] Agop Bedikian,[3,5] Gerald P. Bodey,[3] and Emil J Freireich[3]

Twenty-eight patients with advanced adenocarcinoma were treated with a combination of thymidine, 5-FU, and PALA. PALA (1 g/m^2) was given on the first day, followed on the second day with 30 g of thymidine and 150–300 mg/m^2 of 5-FU given simultaneously through two iv sites. Responses were seen in two of 17 patients (12%) with colorectal adenocarcinoma. One response was complete and one was partial. The gastrointestinal tract and CNS were the sites of dose-limiting toxic effects. Myelosuppression was observed only at 5-FU doses of 250 and 300 mg/m^2. [Cancer Treat Rep 69:611–613, 1985]

The pyrimidine analog, 5-FU, is one of the most widely used antineoplastic agents for the treatment of solid malignancies in humans. Thymidine (TdR), a naturally occurring nucleoside, is a tumor-growth inhibitor when administered exogenously at high concentrations (1). Clinical trials of high-dose TdR have shown activity in patients with acute leukemia and malignant melanoma (2,3). Preclinical studies have demonstrated an enhancement of the antitumor activity of 5-FU by TdR (4,5). Exogenous TdR may divert the biologic effects of 5-FU to RNA metabolism rather than DNA synthesis. Subsequently, the augmentation of 5-FU incorporation into nuclear RNA by TdR was observed by Nayak et al (6).

Clinical pharmacological studies in patients treated with the combination of 5-FU and TdR have shown that TdR can prolong the half-life of 5-FU and also increase its toxicity (7-10). Some antitumor activity was seen in patients with colon and ovarian carcinomas and non-Hodgkin's lymphoma (7).

PALA, a transition-state inhibitor of aspartate carbamoyltransferase, a critical enzyme in the de novo pyrimidine-nucleotide biosynthesis (11), exerts an effect on the incorporation of 5-FU into RNA similar to that of TdR. Preclinical studies conducted by some investigators have indicated potentiation of antitumor activity when PALA is given in combination with 5-FU (12). Clinical responses to the 5-FU and PALA combination were seen in patients with colon cancer (13,14), lung cancer, fibrous histiocytoma (14), and rhabdomyosarcoma (15).

The three-drug combination of TdR, 5-FU, and PALA produced better therapeutic results than either the combination of TdR and 5-FU or 5-FU and PALA in the CD8F$_1$ murine mammary tumor (16,17). Based on the rationale that TdR and PALA given together with 5-FU may have synergistic effects in increasing the antitumor activity of 5-FU through its RNA effects, we tested the combination of these three drugs in patients with advanced solid tumors.

PATIENTS AND METHODS

Patients had histologic proof of malignancy and measurable disease. Recovery from any side effects of previous therapy was a prerequisite for inclusion in the study. Prior therapy with 5-FU or PALA did not affect eligibility. An informed consent was obtained, according to institutional policy, prior to initiation of treatment. Routine pretreatment evaluation and follow-up were performed. Tumor measurements were recorded before each treatment. Response to therapy was evaluated according to standard criteria.

PALA and TdR were obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. PALA was supplied in 10-ml ampules of sterile aque-

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[3] Division of Medicine, Departments of Medical Oncology, Internal Medicine, and Hematology, The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston.

[4] *Present address*: Department of Medical Oncology, American International Hospital, IL.

[5] *Present address*: Medical Affairs Department, Medical Oncology Section, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia.

[*] *Reprint requests to:* Delia F. Chiuten, MD, Department of Medical Oncology, The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, 6723 Bertner Ave, Houston, TX 77030.

ous solution at concentrations of 100 mg/ml; TdR was supplied as a 3% (15 g in 500 ml) solution in isotonic saline at physiologic pH fcr direct iv administration. 5-FU was supplied by Roche Laboratories as 10-ml ampules containing 500 mg of 5-FU/ampule.

The dose schedule was as follows: 1 g/m^2 of PALA was given over 1 hour on the first day, followed on the second day by 30 g of TdR and 150-300 mg/m^2 of 5-FU given simultaneously through two iv sites over 3 hours at 4-week intervals.

RESULTS

Twenty-eight evaluable patients with adenocarcinoma (colorectal, gastric, bronchogenic, pancreatic, and unknown primary) participated in the study. Patient characteristics are shown in table 1.

In the group of 17 patients with colorectal adenocarcinoma, one patient with liver metastasis achieved complete response (CR). This patient had had prior surgery and chemotherapy with a nitrosourea (lomustine) for Dukes D adenocarcinoma of the colon and had developed recurrent disease in the liver only. A computerized axial tomographic scan of the abdomen was negative for liver metastasis after three courses of chemotherapy. CR occurred 24 weeks after the start of treatment and lasted 9 weeks. Another patient achieved a partial response (PR), with a < 50% decrease in liver metastasis. Eight patients (five with colorectal adenocarcinoma, two with gastric adenocarcinoma, and one with bronchogenic adenocarcinoma) had no change in their tumor measurements. The remaining patients had progressive disease. Fourteen of the 17 patients with colorectal adenocarcinoma had received prior chemotherapy with either 5-FU, floxuridine, or tegafur and had become refractory to their treatment.

The gastrointestinal tract and CNS were sites of dose-limiting toxic effects (table 2). One patient receiving 250 mg/m^2 of 5-FU had dysphagia due to severe mucositis. CNS symptoms were drowsiness, headaches, ataxia, and memory impairment and were significant after TdR administration. There were two allergic reactions: a generalized edema and a periorbital edema. There were no drug-related deaths in this study.

TABLE 1.—Patient characteristics

Characteristic	No. of patients
Evaluable	28
Median age in yrs (range): 59 (33-75)	
Prior chemotherapy	24
5-FU	13
Floxuridine or tegafur	8
PALA	1
Other drugs	8
Tumor type (adenocarcinoma)	
Colorectal	17
Gastric	3
Bronchogenic	6
Pancreatic	1
Unknown primary	1

Significant myelosuppression was observed only at 5-FU doses of 250 and 300 mg/m^2 (table 3). Patients who had had prior treatment with nitrosoureas or mitomycin had more myelosuppression than those who had not. The maximum tolerated doses of this schedule were: 1 g/m^2 (PALA); 30 g (TdR); and 300 mg/m^2 (5-FU; 250 mg/m^2 for patients who had previously received nitrosoureas and mitomycin).

DISCUSSION

The rationale for this combination treatment was based on the possibility that synergistic effects of TdR and PALA would increase the antitumor activity of 5-FU by increasing its effects on RNA metabolism. However, results of this phase I-II study suggest that the response rate in patients with colorectal cancer undergoing treatment with this three-drug regimen is not superior to response rates in our previous study of treatment with 5-FU and PALA (13) or other reported studies of 5-FU and TdR (7,9) or 5-FU alone (18). In our previous study, 5-FU and PALA administered weekly to patients with colorectal carcinoma resulted in a PR rate of 15% (13). Response rates ranging from 11% to 25% resulted when TdR and 5-FU combinations were administered to patients with colorectal carcinoma (7,9). Patients given only 5-FU had an overall response rate of 24% (18).

Adding TdR to 5-FU increased the side effects of 5-FU and shifted the dose-limiting toxicity to the gastrointes-

TABLE 2.—Toxic effects*

Toxic effect	No. of courses (%)
Gastrointestinal	
Nausea	29 (42)
Vomiting	15 (25)
Mucositis	14 (24)
Diarrhea	11 (19)
Anorexia	10 (17)
Constipation	1 (2)
CNS	
Drowsiness	16 (27)
Headache	14 (24)
Ataxia	5 (8)
Memory impairment	5 (8)
Visual disturbances	2 (3)
Miscellaneous	
Weakness	18 (31)
Alopecia	4 (7)
Fever	4 (7)
Bleeding	3 (5)
Allergic reaction	2 (3)

*59 (100%) evaluable courses.

TABLE 3.—Hematologic toxicity

	5-FU dose (mg/m^2)			
	150	200	250	300
No. of evaluable courses	10	10	25	11
Median nadir count (× 10^3 cells/mm^3)				
Leukocytes (range)	5.6 (3.5–8.5)	5.6 (2.3–7.3)	3.4 (0.5–9.8)	2.4 (1.2–5.2)
Neutrophils (range)	3.2 (2.2–5.8)	3.1 (1.7–4.0)	1.3 (0–6.7)	0.7 (0.1–2.6)
Platelets (range)	199 (60–341)	189 (142–310)	127 (18–269)	260 (50–391)

tinal tract and CNS. Mucositis became more severe. CNS toxicity was not generally observed in previous studies of treatments combining 5-FU and PALA (13-15). An episode of generalized seizure during infusion of 2 g/m^2 of PALA has been reported by Casper et al (15), but this did not occur in any of our patients. The accentuation of CNS toxicity by the TdR and 5-FU combination was also noted by other investigators (19). Neurotoxicity was dose-limiting in a previously reported phase I evaluation of the PALA, TdR, and 5-FU combination (20).

Our clinical results were not encouraging, possibly because of improper scheduling of the combination or because most of our patients had extensive disease and had failed prior chemotherapy. Eighty-two percent of our patients with colorectal adenocarcinoma had become refractory during prior 5-FU, floxuridine, or tegafur treatment. We do not recommend further clinical evaluation of this drug combination and schedule in previously treated patients with advanced colorectal adenocarcinoma.

REFERENCES

1. LEE SS, GIONVANELLA BC, and STEHLIN JS, JR. Effect of excess thymidine on the growth of human melanoma cells transplanted in thymus-deficient nude mice. Cancer Lett 3:209–214, 1977.
2. CHIUTEN DF, WIERNIK PH, ZAHARKO DS, ET AL. Clinical phase I-II and pharmacokinetic study of high-dose thymidine given by continuous intravenous infusion. Cancer Res 40:818–822, 1980.
3. KUFE DW, BEARDSLEY P, KARP D, ET AL. High-dose thymidine infusions in patients with leukemia and lymphoma. Blood 55:580–589, 1980.
4. SANTELLI G, and VALERIOTE F. In vivo enhancement of 5-fluorouracil cytotoxicity to AKR leukemia cells by thymidine in mice. JNCI 61:843–847, 1978.
5. MARTIN DS, STOLFI RL, and SPIEGELMAN S. Striking augmentation of the in vivo anticancer activity of 5-fluorouracil (FU) by combination with pyrimidine nucleosides: an RNA effect. Proc Am Assoc Cancer Res and ASCO 19:221, 1978.
6. NAYAK R, MARTIN D, STOLFI R, ET AL. Pyrimidine nucleosides enhance the anticancer activity of FU and augment its incorporation into nuclear RNA. Proc Am Assoc Cancer Res and ASCO 19:63, 1978.
7. WOODCOCK TM, MARTIN DS, DAMIN LA, ET AL. Combination clinical trials with thymidine and fluorouracil: a phase I and clinical pharmacologic evaluation. Cancer 45:1135–1143, 1980.
8. KIRKWOOD JM, ENSMINGER W, ROSOWSKY A, ET AL. Comparison of pharmacokinetics of 5-fluorouracil and 5-fluorouracil with concurrent thymidine infusions in a phase I trial. Cancer Res 40:107–113, 1980.
9. VOGEL S, PRESANT CA, RATKIN GA, ET AL. Phase I study of thymidine plus 5-fluorouracil infusions in advanced colorectal carcinoma. Cancer Treat Rep 63:1–5, 1979.
10. AU JLS, RUSTUM YM, LEDESMA EJ, ET AL. Clinical pharmacological studies of concurrent infusion of 5-fluorouracil and thymidine in treatment of colorectal carcinomas. Cancer Res 42:2930–2937, 1982.
11. COLLINS KD, and STARK GR. Aspartate transcarbamylase. Interaction with the transition state analogue *N*-(phosphonoacetyl)-L-aspartate. J Biol Chem 246:6599–6605, 1971.
12. JOHNSON RK, CLEMENT JJ, and HOWARD S. Treatment of murine tumors with 5-fluorouracil in combination with de novo pyrimidine synthesis inhibitors PALA or pyrazofurin. Proc Am Assoc Cancer Res and ASCO 21:292, 1980.
13. BEDIKIAN AY, STROEHLEIN JR, KARLIN DA, ET AL. Chemotherapy for colorectal cancer with a combination of PALA and 5-FU. Cancer Treat Rep 65:747–753, 1981.
14. MESHAD MW, ERVIN TJ, and JOHNSON RK. A phase I trial of combined therapy with *N*-phosphonacetyl-L-aspartate (PALA) and 5-fluorouracil (5-FU). Proc Am Assoc Cancer Res and ASCO 21:341, 1980.
15. CASPER ES, VALE K, WILLIAMS LJ, ET AL. Phase I and clinical pharmacological evaluation of biochemical modulation of 5-fluorouracil with *N*-(phosphonacetyl)-L-aspartic acid. Cancer Res 43:2324–2329, 1983.
16. SPIEGELMAN S, SAWYER R, NAYAK R, ET AL. Improving the antitumor activity of 5-fluorouracil by increasing its incorporation into RNA via metabolic modulation. Proc Natl Acad Sci USA 77:4966–4970, 1980.
17. MARTIN DS, STOLFI RL, SAWYER RC, ET AL. Therapeutic utility of utilizing low doses of *N*-(phosphonacetyl)-L-aspartic acid in combination with 5-fluorouracil: a murine study with clinical relevance. Cancer Res 43:2317–2321, 1983.
18. LIVINGSTON RB, and CARTER SK, eds. Single agents in cancer chemotherapy. New York, Plenum, 1970, pp 195–226.
19. KROOK J, ETZELL P, and GERSTNER P. Potentiation of CNS side-effects of 5-fluorouracil (5-FU) by high dose thymidine. Proc Am Assoc Cancer Res 24:135, 1983.
20. CASPER ES, MICHAELSON RA, KEMENY N, ET AL. Phase I evaluation of a biochemically designated combination: PALA, thymidine, and 5-FU. Cancer Treat Rep 68:539–541, 1984.

Clonogenic and Nonclonogenic In Vitro Chemosensitivity Assays[1,2,3]

Larry M. Weisenthal[4,*] and Marc E. Lippman[5]

We need practical laboratory methods for predicting the chemosensitivity of human neoplasms. Over the past 20 years, numerous investigators have implied or stated with increasing certitude that clonogenic assays are the most valid (or only valid) approach to predictive chemosensitivity testing. We feel that this point of view may have insufficient scientific validity and may be harmful to progress in this area. In addition to well-known technical problems, there are serious theoretical problems with clonogenic assays. These include (*a*) the disruption of normal cell-to-cell interactions, (*b*) the possibility that true tumor stem cells may be largely nondividing (G_0) cells, while cells forming colonies are exclusively dividing cells, (*c*) the possibility that clonogenic cells may largely represent cells which are not true stem cells, and (*d*) the fact that clonogenic assays have the ability to measure cell kill over a narrow log range, while meaningful clinical responses require a multiple-log cell kill. This latter fact mandates the use of unrealistically low drug concentrations to avoid excessive false-positive results. Neither theoretical concepts, direct experimental data, nor clinical correlations support the alleged superiority of clonogenic assays. Clonogenic assays may not be advantageous compared to other more practical methods of estimating the general chemosensitivity of proliferating cells. In contrast, there is a growing body of literature which indicates that early evidence of cell damage in the *entire* tumor cell population may accurately predict for a multiple-log stem cell kill and meaningful clinical response. Future studies should continue to develop and test assays based on alternative methods for detecting cell kill in the proliferating and total tumor cell populations. **[Cancer Treat Rep 69:615–632, 1985]**

It is widely believed that there is a critical need in cancer treatment for a practical technology to allow testing of standard and experimental treatment modalities directly in fresh human cancer specimens rather than in patients. This technology would have the following benefits. (*a*) Clinical care of patients would be immediately improved, because the selection of chemotherapy could be optimized for individual patients. (*b*) Empiric drug screening may be more fruitful if carried out in fresh specimens of human cancer, rather than in established cell lines or subhuman tumor systems. (*c*) Phase II testing of new drugs and phase III comparisons of different treatment modalities in the laboratory (rather than in the clinic) could exponentially improve the efficiency of new drug development. (*d*) Studies of new therapeutic modalities, such as cytotoxicity of human tumor cells mediated by monoclonal antibodies, would be improved. The chief stumbling block preventing the above advances is that a practical assay technology has not been developed and validated.

Attempts to develop tests to predict subsequent in vivo response to cytotoxic therapy in individual patients date back > 40 years for radiotherapy (1,2) and > 30 years for chemotherapy (3). Excluding assays based on the presence of steroid hormone receptors, there have been > 2000 individual clinical correlations published (table 1). Overall, the combined true-positive (TP) accuracy of these tests averages out to be 63%, while the true-negative (TN) accuracy averages out to be 92% (table 1). This is reasonably similar to the accuracy reported for the published correlations based on the estrogen receptor in human breast cancer (4,5).

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[4] Veterans Administration Medical Center, Long Beach, CA, and University of California, Irvine.

[5] Medicine Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

[*] *Reprint requests to:* Larry M. Weisenthal, MD, PhD, Veterans Administration Medical Center, University of California at Irvine—Long Beach Medical Program, Long Beach, CA 90822.

While estrogen receptor determinations have come into widespread use during the last 15 years, the role of in vitro tests to predict chemotherapy or radiotherapy responsiveness is still not established.

Of the many proposed approaches to determine in vitro cell kill, the clonogenic or colony formation types of assay have recently received the most attention for use in predicting clinical responsiveness. In general, cells are plated at a relatively low density in liquid medium (6) or are plated in agar (7,8) and are allowed to grow for four to six doubling times until colonies of > 20 or > 50 cells (depending on the investigator and tumor system) are formed. Differences between control and treated cultures are presumed to relate directly to the fractional kill of clonogenic cells. It has been stated that "assessment of colony formation has been shown to be the only reliable indicator of cell viability after exposure to antineoplastic drugs" (9). Another investigator has stated that "the colony formation assay should continue to be the standard to which other, especially short-term, assays should be compared" (10). Other investigators have stated that clonogenic assays are the only assays which may predict for tumor cure, rather than merely predicting for partial response (11). These investigators have raised technical objections to presently available clonogenic assays, yet have defended the theoretical concept that clonogenic assays are inherently superior to nonclonogenic assays (11,12). Based on personal communications with other investigators, these opinions appear to be very prevalent today. It is probably fair to say that these prevalent opinions have discouraged at least some investigators and reviewers from attempting or funding projects to develop or optimize nonclonogenic types of chemosensitivity assays.

The purpose of this paper is to consider the current status and most promising future directions for in vitro chemosensitivity testing. Previous investigators have reviewed the general area of chemosensitivity testing (13-17). Other reviewers have been critical of the clinical results of clonogenic assays without evaluating the concepts on which these assays are based (11,18,19). We will examine the evidence which has been cited to support the concept that clonogenic assays represent the "only reliable" approach to in vitro chemosensitivity testing. We will review the published data regarding the accuracy of the different types of assays in predicting for clinical responsiveness. Finally, we will discuss the practical strengths and weaknesses of the different classes of assays and make recommendations concerning future directions for work in this critical area of cancer treatment research.

HISTORICAL PERSPECTIVE

It is worthwhile to consider the historical reasons why clonogenic assays became popular for assessing cytotoxic damage in tumor cells. In the early 1960s, much work was done to assess the radiation sensitivity of established tumor cell lines in vitro. The differences in apparent radiation sensitivities of the cell lines under study were small and it was desirable to assess cell killing over a multiple-log range. For these reasons, maximum assay precision was desirable. Two of the

TABLE 1.—Summated clinical correlations of predictive chemosensitivity assays[*]

	Assay type				% specificity†	% sensitivity‡	Predictive accuracy§ (positive/negative)
	TP	TN	FP	FN			
Tumor shrinkage¶	57	68	35	8	66	88	62%/89%
Cell metabolism[**]	47	62	45	1	58	98	57%/98%
Cell morphology††	36	39	45	11	46	77	44%/78%
Membrane function‡‡	45	87	19	2	82	96	70%/98%
DNA precursor incorporation§§	148	297	74	20	80	88	67%/94%
Clonogenicity¶¶	215	712	110	62	87	78	66%/92%
Total	548	1265	328	104	79	84	63%/92%

[*] Data listed may not be comprehensive, but should be representative. FP = false-positive; FN = false-negative.

†Specificity = TN/(TN + FP).

‡Sensitivity = TP/(TP + FN).

§Predictive accuracy for a positive assay = TP/(TP + FP); predictive accuracy for a negative assay = TN/(TN + FN).

¶Subrenal capsule assay (ref Nos. 92, 138, and 139).

[**] Ref Nos. 3 and 144-147.

††Ref No. 95.

‡‡Ref Nos. 38, 104, 105, 140-143, and 148.

§§Ref Nos. 107, 124, 125, and 149-162.

¶¶Ref Nos. 92 and 163-180.

methods used to assess cell killing were (*a*) the back extrapolation of the mass culture growth curve (20) and (*b*) the Puck-Marcus clonogenic assay (21). The latter assay was preferred by radiobiologists because (*a*) there was sometimes difficulty in choosing which portion of the growth curve to extrapolate, and (*b*) it was felt that measuring the reproductive capacity of individual cells might have some unstated advantages compared to measuring growth in the total cell population (20). Notwithstanding these concerns, it is noteworthy that measurements of cell survival by both techniques were remarkably similar (20). This concordance is especially impressive to us when viewed in the light of the large coefficients of variation which are typically seen with today's clonogenic assays in human tumors (22). Whether clonogenic assays have been of help in explaining radiosensitivity differences between different types of tumors is a question which might be best addressed by radiobiologists (23-25).

Unlike the problem radiobiologists face in making precise comparisons between multiple degrees of log kills in cell lines which appear to be exquisitely sensitive to radiation, chemobiologists are faced with the more basic problem of studying compounds which may not produce any effective degree of cell killing at all. There are good theoretical reasons to suppose that quantifying cell growth in mass culture systems may be comparable, if not identical, to quantifying colonies in clonogenic assay systems. Figure 1 shows a hypothetical case in which the pretreatment cell population consists of both clonogenic and nonclonogenic cells. Following drug treatment, a certain percentage of clonogenic cells may be killed outright, a certain percentage may receive a degree of DNA damage which is not immediately lethal but which becomes lethal after one or more cell divisions, and a certain percentage may be unharmed. If one defines a clonogenic cell as being the ancestor of proliferating cells measured five generations after the first division of the clonogenic cell (the time required to form a 50-cell colony), then measurements of the number of proliferating cells at appropriate times should relate directly to the number of surviving clonogenic cells. This example was chosen for simplicity, but other randomly selected examples would also illustrate the same point. It will be our continuing thesis that measurements taken of the entire tumor cell population (instead of only the clonogenic cell population) may be of great practical value in chemobiology.

Notwithstanding our own point of view, it is a fact that clonogenic assays were considered to be the gold standard assay method in radiobiology by the early 1970s. Other investigators began to use comparable assays in studies of in vitro sensitivity to drugs and obtained positive clinical correlations with animal neoplasms (26). Still other investigators devised techniques for cloning human tumors in soft agar (7,8,27-29). Roper and Drewinko (30) reported that the results of clonogenic assays in established cell lines did not agree with the results of nonclonogenic assays. Steel (31) described evidence supporting a stem cell model of human tumor growth. Salmon et al (32) described positive clinical correlations in human neoplasms with a soft agar clonogenic assay. These later three publications stimulated enormous interest in using clonogenic assays for clinical drug testing and preclinical drug screening.

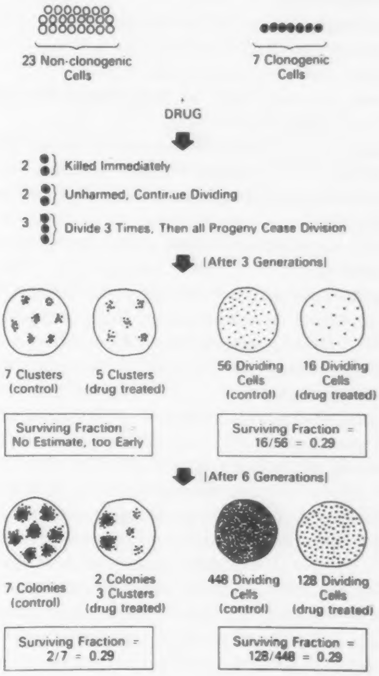
23 Non-clonogenic Cells
7 Clonogenic Cells
DRUG
2 Killed Immediately
2 Unharmed, Continue Dividing
3 Divide 3 Times, Then all Progeny Cease Division
(After 3 Generations)
7 Clusters (control)
5 Clusters (drug treated)
56 Dividing Cells (control)
16 Dividing Cells (drug treated)
Surviving Fraction = No Estimate, too Early
Surviving Fraction = 16/56 = 0.29
(After 6 Generations)
7 Colonies (control)
2 Colonies 3 Clusters (drug treated)
448 Dividing Cells (control)
128 Dividing Cells (drug treated)
Surviving Fraction = 2/7 = 0.29
Surviving Fraction = 128/448 = 0.29

FIGURE 1.—Colony counting vs counting of proliferating cells to determine fractional kill of clonogenic cells. This hypothetical example shows a situation in which a total of 30 cells contain 23 nonclonogenic cells and 7 clonogenic cells. Following exposure to a cytotoxic agent, 2 clonogenic cells are killed immediately, 2 are unharmed and continue to divide, and 3 divide 3 times, before all progeny cease division. If clonogenic cells are defined by their ability to give rise to progeny which are themselves capable of sustained cell division, then measurements of the numbers of proliferating cells several generations following drug exposure [by cell counting, ^{3}H-thymidine (TdR) incorporation, or some other means] should relate to the surviving fraction of clonogenic cells in the same manner as counting colonies. This model makes a No. of obvious assumptions, which are not out of line with the larger No. of assumptions which must be made with standard clonogenic assays (discussed in text). Colonies are defined as having > 50 cells and clusters are defined as having < 50 cells.

TECHNICAL PROBLEMS WITH CLONOGENIC ASSAYS

Although clinical correlations with clonogenic assays have appeared to be promising (table 1), a number of technical problems became evident:

(*a*) clump artifacts (19,34,35,39);
(*b*) artifactual growth perturbation resulting from simple manipulations of the culture plates (34);
(*c*) drug exposure reducing nutrient consumption by nonclonogenic cells and artifactually increasing the size and number of surviving clonogenic cell colonies (40);
(*d*) difficulties in colony counting (181);
(*e*) poor evaluability rates (22,33);
(*f*) large coefficients of variation (22);
(*g*) poor reproducibility of test results between different laboratories (120);
(*h*) large numbers of cells required, frequently possible only to test small numbers of drugs (22,34);
(*i*) several types of tumors that cannot be tested at all (34);
(*j*) long time period before assay can be evaluated (182); and
(*k*) time- and resource-consuming (expensive) (181).

These technical problems have been previously discussed by other investigators, and we will not discuss all of them here. However, two of these problems deserve special emphasis.

The poor cloning efficiencies of most specimens create serious problems. First, the majority of assays from most investigators are not evaluable for chemosensitivity (19,33,34). Second, the problems of distinguishing true colonies from cell aggregates and other artifacts become particularly important in experiments in which very few true colonies are present. Although the presence of clump artifacts has been previously recognized (11,19,22,34,35), this problem is probably more serious than has been generally acknowledged. A paper by Bertoncello et al (35) is very instructive with regard to this problem. These investigators received 99 cytologically positive specimens of human ovarian cancer. Of this total, 12% were impossible to dissociate to a single-cell suspension, 3% became contaminated, 14% had low viability, and 29% had insufficient numbers of cells for plating. Forty-one percent of their specimens were adequate for culture, and 58% of their cultured specimens (23% of the cytologically positive specimens received) exhibited growth in a soft agar system. However, these investigators further defined the type of growth seen as either Type I (clusters of < 40 cells or colonies of < 150 μm, with evidence of necrosis) or Type II (colonies of good quality, containing only healthy, light-refracting cells). Small aggregates of degenerating cells were not scored at all in their study, but a photograph of one of these nonscored aggregates (35) appears to be comparable with what other investigators have scored as a true colony (36). Bertoncello et al (35) found that only 22% of their successful cultures (5% of the original cytologically positive specimens received) exhibited Type II growth. The experience of one of us (L. M. Weisenthal) indicates that Type II growth is generally seen on technically successful assays performed on established cell lines. Type II growth, however, is infrequent with human tumor biopsy specimens, which have truly been dissociated to single cells. Based on visits to other clonogenic assay laboratories and on observing slides of typical colonies (best colonies?) projected at meetings, we feel that what typically passes for a colony in many laboratories might be considered to be either a Type I colony or no colony at all by Bertoncello et al. Selby et al (11) have noted that the presence of clumps or other artifacts is probably responsible for unrealistically flat radiation survival curves. These investigators (11) and others (37) have stated that these artifacts probably exist also in chemosensitivity experiments. This may create a bias in these assays to predict for drug inactivity. This could produce an impressive TN rate in clinical correlation studies in which most patients have tumors which are not sensitive to any chemotherapy (38).

The practices of surveying Day-0 plates to ensure the presence of a good single-cell suspension or subtracting Day-1 colony counts from the final colony counts do not obviate the important problem of clump artifacts. The National Cancer Institute has sponsored an extensive study of the clonogenic assay system, particularly as applied to new drug screening (22). Four highly experienced laboratories tested a number of agents at a 10-μg/ml continuous exposure. Sixty percent to 65% of specimens meeting quality control standards were not sensitive to spirogermanium, sangivamycin, and homoharringtonine (38). In the experience of one of us (L. M. Weisenthal), virtually all neoplastic and normal human cells have been sensitive to these drug exposures, using an assay system which is considered to be less sensitive than the clonogenic assay in detecting cell kill (38). This suggests that most of the clonogenic assay results were FN, probably because clump artifacts are not reduced by exposure to most drugs. Alley and Lieber (39) used filtration through a 70-μm mesh to prepare single-cell suspensions. The median Day-1 colony count for evaluable assays was 8, and a *net* increase of 30 colonies was required by these investigators for an evaluable assay. Despite these procedures to eliminate clumping artifacts, most assays produced FN results, compared to identical assays in which nonviable artifacts were distinguished from viable cell groups by means of tetrazolium dye staining (39). Tetrazolium identifies living cells by means of changing color in the presence of a metabolically active cell. It must be cautioned that even this technique may underestimate clumping artifacts, since some cell clumps may remain metabolically active during the culture period, yet not represent proliferating cell colonies. Another means of attempting to identify plates with clump artifacts has been to treat some plates with extremely high concentrations of toxic compounds, eg, 600 μg/ml of sodium azide or 100 μg/ml of mercuric chloride (22). Such agents could potentially destroy and lyse viable cell clumps, in addition to the desired effect of preventing clonogenic cells from forming colonies. This could falsely reassure the investigator that the features being counted were truly colonies and not clump artifacts. A better positive control would be to irradiate several control plates with 20 Gy of photon energy. This should effectively eliminate colony formation (11,25,37).

It is dangerous to draw conclusions regarding both chemosensitivity and cell biology from experiments in which inadequate quality controls raise the possibility that the results reported may reflect differences in clump artifacts, rather than true differences in colony formation. Peer review in this area must become much more demanding. We would suggest the following minimum standards for an acceptable assay: (*a*) maximum Day-0 colony count of ten (39); (*b*) net increase of at least 70 colonies (22); (*c*) colony counts in control cultures to exceed colony counts in irradiated (2000 rad) positive control cultures by at least 70; and (*d*) accepting 40% as the upper limit for the coefficient of variation of the mean colony count of the control group. The routine use of tetrazolium to distinguish viable from nonviable cell groups is practically and theoretically attractive (39). However, one concern is that true colonies might form by Day 7 and yet the cells in the colony may die by Day 14, due to nutrient inadequacies (40). This potential problem requires further study.

As a final and sobering technical note, it may be that the search for better nutrient additives to improve plating efficiencies may turn out to be a very difficult problem, perhaps defying satisfactory solution. A large number of additives and culture modifications have been proposed (8,41–47). Several investigators, however, did not find that more complex media produced consistent improvements compared to more simplified media (38,46,48). Growth of hormone-dependent tumors apparently may be improved somewhat by adding appropriate hormone supplements (43). As will be discussed later, however, it is not at all clear that it is the stem cell population whose growth is stimulated by these hormone additives.

With these many technical problems, it is logical to consider whether it might not be more productive to use other types of assay systems to predict drug sensitivity. We will now examine the evidence which has been cited to support the superiority of clonogenic assays.

EVIDENCE SUPPORTING THE SUPERIORITY OF CLONOGENIC ASSAYS OVER NONCLONOGENIC ASSAYS

There are three main lines of evidence which have been advanced to support the superiority of clonogenic assays. First, there are purely theoretical arguments. Second, there have been experiments performed comparing clonogenic assays with nonclonogenic assays in established cell lines. Third, there is an increasing quantity of clinical correlation data. Each of these three lines of evidence will now be considered.

Theoretical Arguments

Human tumors consist of a mixture of dividing and nondividing cells, with most cells often being nondividing (50). Of the nondividing cells, some are irreversibly nondividing (or permanently G_0), while others are reversibly nondividing (or temporarily G_0) (31,51). Of the dividing cells, some are thought to have only a limited division potential (before the cell progeny become permanent G_0 cells), while others are postulated to have an unlimited division potential. These latter cells have been labeled tumor stem cells (31) and are proposed to be the most important tumor cell subpopulation by those investigators promoting clonogenic assays.

To accept the theoretical superiority of clonogenic assays, a number of important assumptions must be made. First, it must be assumed that clonogenic cells in vitro are representative of the most relevant stem cells in vivo. (In the following discussion the term stem cells refers to an in vivo tumor cell population, while the term clonogenic cells refers to an in vitro tumor cell population.) Second, one must assume that the stem cell population is the only relevant tumor cell target for chemotherapy. Third, one must postulate that the chemosensitivities of dividing and nondividing stem cells are different from the chemosensitivities of dividing and nondividing non-stem cells, respectively.

The assumption that clonogenic cells are the most relevant tumor cells is highly questionable (38,52). Clonogenic cells in a short-term assay are, by definition, a cell population with a 100% growth fraction. Tumor cells in vivo are a mixture of dividing cells and temporarily nondividing (G_0) cells, as suggested by studies on tumor growth kinetics and recruitment of G_0 cells (53–56). It has often been speculated that it is the failure to kill G_0 cells which is partly responsible for the ineffectiveness of chemotherapy in human neoplasms (57). Clonogenic assays are probably not capable of estimating cell kill in this extremely important G_0

population. Even if the cell culture conditions of the assay were sufficient to recruit these G_0 cells into the cycling population (so they could grow into colonies), this would not mimic the clinical situation. In vitro, recruitment of G_0 cells would presumably arise by virtue of supplying nutrients to cells which had been starved in vivo (58–60) or by removing the tumor cells from the presence of some inhibitory regulatory factor(s) present in vivo (53,56). Thus, recruitment would take place at the time when the cells were first placed in culture following tumor dissociation. That this is the case is supported by studies showing very high ^{3}H-TdR suicide indices in standard clonogenic assays in several types of human tumors (61). In a standard clonogenic assay, this recruitment would take place at a time when the cells either contained cytotoxic drug or possibly before they were able to repair what might only have been sublethal damage had they remained nondividing (62,63). In the clinical situation, recruitment most likely occurs after tumor debulking induced by drugs or from tumor-induced angiogenesis. As contrasted with the situation of in vitro recruitment, it is *far* more likely that the G_0 cells in vivo will remain in G_0 until long after the effects of the drug have passed.

It cannot even be assumed that clonogenic assays measure stem cells at all. In studies of normal murine hematopoiesis, Suda et al (64) have found that (*a*) stem cells appear to have long and variable G_0 periods compared to cells already committed to terminal differentiation, and (*b*) activation of stem cells appears to be purely random (stochastic). These cells are *not* recruited from G_0 phase merely by plating them in clonogenic culture. They are resistant to 5-FU in vivo and have a low ^{3}H-TdR suicide index in vitro. This contrasts with cells committed to differentiate, which are sensitive to 5-FU in vivo and have a high ^{3}H-TdR suicide index in vitro [as do human tumor clonogenic cells (61)]. A differentiating tumor cell hierarchy may or may not recapitulate a differentiating normal cell hierarchy (65). However, Karp et al (49) have recently presented data in human multiple myeloma which strongly imply that most tumor stem cells are G_0 cells and are *not* recruited to divide when placed in soft agar culture unless certain stimulatory factors are added to the cultures. We cannot be sure that such stimulatory factors, once identified for a given type of tumor, are sufficient to recruit all or even most of the putative stem cells or whether they are only serving as growth factors for committed non-stem cells. The point is that proponents of the stem cell theory should not assume that clonogenic assays measure all or even any stem cells.

As an additional theoretical point relating to the possibility that clonogenic assays may not measure stem cells at all, Mackillop et al (65) have discussed further evidence that many of the colony-forming cells in vitro may, in fact, be only transitional cells incapable of sustaining tumor growth in vivo. If valid, this concept would indicate that traditional one-stage clonogenic assays are not necessarily more reliable indices of stem cell killing than are other in vitro assays measuring the survival of proliferating cells. The above investigators have attempted assays which measure self-renewal of colonies (the formation of secondary colonies in cultures replated from disaggregated primary colonies). In acute leukemia, cell survival in a one-stage clonogenic assay did not correlate with clinical outcome, but colony self-renewal did appear to be of some biological relevance (66). It is, however, most unlikely that complicated assays based on the survival of disaggregated and replated clonogenic cells would ever prove to be practical for clinical application.

The argument that only clonogenic assays may predict for tumor curability also ignores the important problem of resistant tumor cell subpopulations. In well-characterized animal tumor systems, Skipper and Schabel (67) have found that many drugs can produce multiple-log cell kills of stem cells, yet have a negligible effect on tumor size and no effect on the survival of the animal. Similar findings have been reported by Stephens et al (68). Responsive but incurable tumors appear to survive chemotherapy because of the presence of tiny populations of resistant clonogenic cells (less than one cell per 10,000). Since it is highly doubtful that the typical clonogenic assay performed on human tumors will be capable of detecting a $>$2-log tumor cell kill, there is little hope that these assays may detect the presence of these resistant tumor cells. This again indicates that there is no reason to postulate that clonogenic assays should be superior predictors of tumor curability with most drugs than other general assays of chemosensitivity in proliferating cells. In the tumor models of Skipper et al, it appears that clonogenic assays would be almost irrelevant if a 5-log cell kill was predictive only for the most marginal of tumor responses. It appears that these assays, at least in some tumors, have an overly sensitive *threshold* (ability to detect *any* degree of cell kill) and an insufficiently sensitive *range* (ability to detect *multiple logs* of cell kill). Some investigators have tried to circumvent this problem by testing *subclinical* drug concentrations to avoid excessive numbers of FP assay results (69). This introduces yet another degree of artificiality into the system and strikes us as being particularly undesirable, given that drug transport into the cell may often be an important determinant of drug sensitivity. Drug transport may be highly dependent on extracellular drug concentration. In this context, it is plausible that an assay system with a less sensitive threshold or a more sensitive range could have equal or superior predictive value for the clinical effectiveness of cytotoxic agents.

Stated concisely, the major theoretical problems with clonogenic assays are as follows:

(*a*) Single-cell suspension results in loss of normal cell-cell interactions (188).

(*b*) Cells already committed to differentiation may form colonies in vitro (65).

(*c*) Reversibly nondividing (G_0) cells are probably not assayed [all cells assayed are actively proliferating and are probably the tumor cells which are *most sensitive* to chemotherapy (38,184)].

(*d*) True stem cells (cells with capacity for self-renewal) in vivo may be mainly G_0 cells; cells committed to differentiation may be immediately recruited in vitro, while true stem cells may be only slowly and randomly recruited in vitro (64) or not recruited at all (49).

(*e*) If (*c*) and (*d*) are true, then clonogenic assays may not even test stem cells; furthermore, nonclonogenic assays which may measure drug effects in G_0 nonclonogenic cells may more accurately predict for drug effects on stem cells than do clonogenic assays which would measure drug effects almost exclusively on actively proliferating non-stem cells.

(*f*) The limited (1-2-log cell kill) range of clonogenic assays in human tumors is insufficiently sensitive in predicting for drug sensitivity (67,68), so that *subclinical* drug concentrations must be used to avoid excessive FP results (69); this artifactual adjustment may not reliably be a model for the actual drug-cell interactions in vivo.

We conclude that clonogenic cells in vitro may theoretically not be a valid counterpart to therapeutically relevant stem cells in vivo. To the extent that clonogenic cells accurately reflect the general chemosensitivity of the bulk of the proliferating tumor cells in vivo, clonogenic assays may be theoretically most valuable for selecting agents which may halt short-term disease progression or produce temporary responses. To the extent that the chemosensitivity of G_0 stem cells may be different from the chemosensitivity of clonogenic cells, clonogenic assays may be theoretically incapable of detecting the activity of uniquely needed agents active against G_0 cells and required for long-term disease control. Thus, nonclonogenic assays which are capable of detecting cell kill in nondividing cells could offer unique advantages in clinical chemosensitivity testing and drug screening.

It should also be noted that the stem cell population may not be the only relevant target population for chemotherapy. Cells with a limited division potential may be important targets for chemotherapy at a time when only one or two tumor doublings may be lethal. Permanently nondividing G_0 cells may even be important targets for chemotherapy if they contribute to morbid phenomena such as cancer-related cachexia, immunosuppression, and paraneoplastic syndromes. Selby et al (70) have raised yet another provocative possibility. The hormonal therapy of breast cancer is an obvious case where a most useful therapeutic effect is obtained with a form of treatment which, in all probability, does *not* act on the tumor stem cell compartment (70). These investigators go on to speculate that tumor debulking may leave behind "a population of more actively dividing tumor stem cells which are more sensitive to chemotherapy." Presumably, this argument could also apply to chemotherapy. Agents which were effective in killing tumor non-stem cells might also be expected to result in the recruitment of stem cells and make them more vulnerable to chemotherapy. In summary, it would appear that stem cells are not the only relevant targets for chemotherapy.

There is no compelling reason to believe that the chemosensitivity of a clonogenic cell should be remarkably different from that of a nonclonogenic proliferating cell. This issue has been addressed in normal gastrointestinal, testicular, and bone marrow tissues, although not in tumor cell systems, to our knowledge. One problem with stem cell studies on gastrointestinal epithelium is that cells may migrate and be sloughed away before morphologic evidence of cell damage has become apparent. Another problem is that some investigators have attempted to study differences between the cell damage created in different cell populations by examining drug doses which produced clinically irrelevant (< 1 log) levels of stem cell kill (71). Because of these and other problems, it is difficult to draw valid inferences from these studies which may be related to tumor cell populations. The limited data which relate to this issue suggest that differences in chemosensitivity between stem cells and non-stem cells are small, compared to the differences between dividing and nondividing cells present in the same tissues (72). Where differences between stem cells and non-stem cells have been shown to exist, they appear to be related more to differences between the percentage of cells which are actively proliferating, rather than due to intrinsic chemosensitivity differences independent of proliferative state (64,73). The importance of this point is that there are easier, faster, and cheaper assays to measure cell kill in proliferating cells (TdR incorporation, growth of tumor microspheroids, etc). In the absence of data demonstrating important chemosensitivity differences between stem cells and non-stem cells which are not merely related to differing rates of cell proliferation, it does not seem productive to insist that clonogenic assays are more valid than other assays which measure cell kill in proliferating cells.

There are two final theoretical arguments which have been cited to support the alleged superiority of clonogenic assays. The first is the possibility that some drugs may cause a cell cycle progression delay out of proportion to cell lethality (74). We have discussed this problem previously (75). The preponderance of published data did not suggest that cell cycle progression delays out of proportion to cell kill are a major problem with most drugs at clinically relevant levels of cell kill. Additionally, clonogenic assays are also not immune to potential artifacts which could be caused by this problem if it does, in fact, exist. More recently, other investigators have presented further evidence that important cell cycle delays occur only in cell populations which are truly drug-sensitive (76,77). The problem with all of these studies lies in the definition of cell kill. This is generally defined by the results of a clonogenic assay. As will be discussed later, clonogenic assays may give *highly* variable results, depending on the assay conditions.

The final theoretical argument which has been used to support clonogenic assays is that drugs may cause delayed death, which occurs in a tumor clone only after several generation times following drug exposure. We also discussed this point elsewhere (75). We noted that it has not been demonstrated that the fraction of cells undergoing delayed death is large compared with the fraction of cells undergoing early death at clinically relevant levels of cell kill. In a particularly elegant study, Ehmann and Wheeler (78) examined the fate of single proliferating cells exposed to carmustine. At drug concentrations which produced only minor (< 1-log) differences in cell kill, most cells completed several mitoses before a part or all of the progeny succumbed to the inherited damage. However, at drug concentrations which produced a kill of 2 logs of clonogenic cells, most cells had gross evidence of morphologic damage *before* the first cell division and 90% of the cells died within the first 4 days (78). As noted above, a clinically meaningful drug effect probably requires a multiple-log clonogenic cell kill [which may sometimes be predicted in vitro by observing lesser degrees of cell kill at a *subclinical* drug concentration (69)]. Therefore, *early* evidence of cell damage observed in a short-term assay may very well predict accurately for a multiple-log tumor cell kill and tumor responsiveness. Conversely, the absence of early damage may accurately predict for inconsequential degrees of cell kill and tumor nonresponsiveness.

We conclude that there are no compelling theoretical arguments to support the premise that clonogenic assays are inherently superior to nonclonogenic assays. Several of the above theoretical arguments even support the concept that nonclonogenic assays measuring early evidence of cell damage might be more useful than clonogenic assays for predicting the behavior of the entire tumor cell population and tumor mass.

Assay Comparisons

A number of investigators have made direct head-to-head comparisons of clonogenic and nonclonogenic assays. These results have often been cited as support for the superiority of clonogenic assays. We will discuss the most important of these papers individually. First, however, we will make some general comments.

In the papers to be discussed, clonogenic assays are compared to one or more nonclonogenic assays after exposure of the cells to fixed drug concentrations. One major problem with most of these papers is that the results of the clonogenic assays are considered to be the correct result (the "gold standard"), while the nonclonogenic assays are considered to be incorrect if they do not agree with the clonogenic assay. This practice ignores several possibilities. First, the results of the clonogenic assay may simply be incorrect and the results of the nonclonogenic assay may be more correct. The results of clonogenic assays may be highly variable, depending on the time when the cells are plated out in single-cell suspension following drug exposure and on several other factors relating to assay conditions (74,80). Second, it is possible that different assays may be equally useful in predicting in vivo tumor response or cure but may require *different* in vitro drug concentrations or conditions of drug exposure to predict accurately (75,79). These data caution that assays require individual calibration and that one should not draw sweeping conclusions from the simple comparison of drug effects at fixed drug concentrations. The only correct comparison is to the results of a clinical trial.

In addition to the above considerations, most investigators attempting assay comparisons have failed to take into consideration a number of critical factors of importance to the success of the nonclonogenic assays being studied. The papers most often cited will now be individually discussed.

Bhuyan et al (81) compared a clonogenic assay with dye exclusion, ^{51}Cr release, and loss of cell attachment to the culture plate as alternative indices of cell death after drug exposure. The clonogenic assay was found to be the most sensitive approach. However, the investigators did not compare these assays to in vivo antitumor response, nor did they consider the possibility that the use of higher drug concentrations may have allowed for a valid assessment of the differences in activity between the different drugs in the nonclonogenic assay systems. In addition, there were methodologic problems in their use of the nonclonogenic assays. All of the nonclonogenic cell viability tests were made immediately after drug exposure. Since these assays all measured some aspect of cell membrane function, and since loss of membrane function is likely to be a late event following a lethal insult to the cell nucleus, it is quite possible that delaying the assays for several days following drug exposure may have resulted in higher estimates of cell killing with the alternative techniques. We found that the survival of HL-60 promyelocytic leukemia cells decreased from 38% to 17% to 0% when assessed 2, 4, and 6 days after a 1-hour exposure to doxorubicin by means of a dye exclusion assay (75). Other investigators (10,30,82,83) may well have underestimated cell lethality because of an insufficient waiting period following a cytotoxic insult.

Roper and Drewinko (30,84) also compared a clonogenic assay with dye exclusion and ^{51}Cr release and examined cell growth in mass culture, the labeling index, and a test which they referred to as the scintillation index. Compared to the clonogenic assay, all four of the latter tests seriously underestimated cell killing caused by bleomycin and carmustine, while the cell growth assay somewhat overestimated cell killing caused by doxorubicin. As in the above-described reports, Roper and Drewinko did not compare these assays to in vivo antitumor responses, nor did they test the hypothesis that using different drug concentrations for each test might have revealed differences in the activity between the different drugs which might have accurately reflected differences between their relative activities against the tumor cells. These investigators also failed to take into account certain important technical considerations which may have a profound influence on the assay results. For example, in their comparison of a clonogenic assay versus the mass culture cell growth assay, they quantified the latter assay by measuring differences in the initial slopes of the growth curves. This may not be the best way of expressing a result for this assay. Following exposure to a cytotoxic agent, cell lines characteristically exhibit a growth lag (secondary to the loss of lethally damaged cells and possibly to a cell cycle delay in the sublethally damaged cells), followed by a resumption of logarithmic growth, the curve of which parallels that of the log phase of the control cultures. By extrapolating either the control or experimental curve and calculating the ratio of the experimental cell numbers over the control cell numbers (20,85), one may obtain a theoretically more accurate estimate of cell kill than by measuring differences in the initial slope of the growth curves (fig 1).

Additionally, the scintillation index assay of Roper and Drewinko (84) measured ^{3}H-TdR incorporation, but expressed the results as a function of radioactive counts per viable (adherent) cell. A much more relevant question would be how much the radioactivity was reduced in the entire cell population (fig 1). A drug could kill 99.9% of the tumor cells, yet not alter the rate of DNA synthesis in the 0.1% of the cells surviving treatment. Other investigators have shown good correlation between clonogenic assays and ^{3}H-TdR assays which are based on this latter approach (86-89).

Technical pitfalls which were not considered by Roper and Drewinko (30) in studies purporting to illustrate the uselessness of dye exclusion assays were discussed by us in a previous publication (75).

Most recently, Rupniak et al (83) compared a clonogenic assay with dye exclusion and autoradiographic (labeling index) assays. These investigators also did not consider the technical pitfalls for dye exclusion assays which we described (75). Likewise, the labeling index (^{3}H-TdR-labeled cells per total cells) is also artifactually depressed by continued proliferation of surviving cells and early disintegration of killed cells. An alternative ^{3}H-TdR autoradiographic assay (38,89) to correct for these artifacts has been described. This assay has given results comparable to those in a clonogenic assay (89; unpublished observations).

There have been few attempts to compare the accuracy of clonogenic assays with the accuracy of nonclonogenic assays in predicting for in vivo tumor chemosensitivity. A number of studies have shown that different types of clonogenic assays (in vitro and in vivo) have results which are generally, but not always, comparable (68,80,90,91). In one published study comparing a clonogenic assay with the subrenal capsule (SRC) assay (a nonclonogenic assay which is carried out in vivo and which measures cell damage in the total cell population), the two assays were 29% concordant for drug sensitivity and 83% concordant for drug resistance (92). Clinical correlations showed that the SRC assay had three TP predictions of tumor responsiveness, no FP predictions, 16 TN predictions, and four FN predictions. The clonogenic assay had no TP predictions of tumor responsiveness, no FP predictions, ten TN predictions, and no FN predictions. The SRC assay was evaluable 89% of the time and the clonogenic assay was evaluable 42% of the time. The above data are preliminary, but do not allow us to conclude that one type of assay has superior validity compared to the other.

Considering the above points, the direct experimental evidence purporting to illustrate the superiority of clonogenic assays is unconvincing.

The "Proof of the Pudding": Clinical Correlations

As noted in the preceding discussion, it may be misleading to conclude too much about the potential clinical usefulness of proposed in vitro assays based on theoretical considerations or on comparative in vitro testing at a drug concentration fixed for all of the assays. The most valid comparisons concern how well, and at what price, assays predict clinical responsiveness. Unfortunately, only the limited study of Bogden and Von Hoff (92) made a comparison of different tests in a uniform patient population. Also, it is difficult to compare different tests, since criteria for correlations are different for different investigators. With these reservations, we will attempt to review the assays shown in table 1, grouped according to the major principles on which the assays are based.

Assays Measuring Changes in Cell Metabolism

These assays were the earliest reported tests for predicting clinical response to chemotherapy. Based on the correlations noted in table 1, it would appear that these assays may have some validity in predicting for chemotherapy nonresponsiveness. The basic premise of these assays is that short-term exposure to high concentrations of cytotoxic drugs may produce an early inhibition of cell metabolism. The absence of this effect implies a low probability of drug effectiveness. Due to the rapidity and simplicity of these tests, it would be worthwhile to test this hypothesis further. Recently, Kurnick et al (93) have described a practical and simple metabolic assay based on the measurement of $^{14}CO_2$ released into the gas phase of culture bottles containing ^{14}C-glucose. It is quite probable that these tests may turn out to have some validity for specific types of drugs or tumors while lacking predictive validity for others. A simultaneous testing of new drugs in both tumor and normal tissue in a metabolic assay has been suggested as a screening test to detect agents with selective toxicity against tumor cells (94).

Assays Measuring Gross Cell Damage in the Entire Cell Population

Cell damage assays have been used to predict responsiveness to radiotherapy in cervical cancer since the middle 1940s (1,2). The tumors are biopsied 1 week following radium implants and routine histologic preparations are screened by a pathologist for evidence of tumor cell damage and changes in keratinization. This crude test has shown a rather remarkable correlation with long-term disease-free survival (1,2). Patients with a positive assay result had a sixfold greater probability of surviving 5 years than patients with a negative assay result. Considering that a radiotherapy cure requires a multiple-log cell kill, it is interesting that a test probably not capable of detecting more than a 1-2-log reduction in a population of cells which is mostly nondividing would have any predictive validity at all (2). If one considers the added technical problems associated with identification of tumor cells versus normal cells and variations in tumor sampling in hypoxic areas versus well-perfused areas, the predictive validity is even more surprising. The positive predictive value of this assay supports our hypothesis (stated earlier) that very gross early evidence of cell damage in the whole tumor may correlate with a multiple-log cell kill in that population of tumor cells which is responsible for sustained tumor growth. These studies were particularly impressive because the results were based on long-term survival and local disease control of easily visible tumors and are less subject to observer bias and variability than are studies based on the more nebulous criteria of tumor responses. The studies provide strong evidence that it is not necessary to study the clonogenic tumor fraction to obtain useful information regarding long-term tumor control. An early in vitro chemosensitivity trial based on morphologic changes gave only modestly positive results (table 1; 95). It is possible that these marginally useful correlations were due to an inherent inaccuracy in the technique itself, but it is also possible that the investigators failed to use drug exposures appropriate to their technique or that they failed to prevent or distinguish fibroblast growth in their cultures. Other investigators based treatment of ovarian cancer on the results of similar in vitro cell morphology assays, with a survival advantage for the "treated by assay" group seen in a nonrandomized study (96), but without a significant difference in survival in a randomized study (97). The above data do not permit a definitive conclusion concerning the potential validity of morphology assays for in vitro chemosensitivity testing, but these assays do have the inherent limitation of being subjective and nonquantitative.

The in vivo SRC assay of Bogden is a unique assay based on the principle of short-term morphologic damage to the entire tumor cell population. This assay is considerably more expensive than most short-term in vitro assays, but probably has the highest evaluability rate for solid tumors of any assay described in the literature (98,99) and has given promising clinical correlations (table 1). Histologic examination for evidence of tumor cell damage appears to be a more reliable end point for this assay than changes in tumor size (100-103). With the histologic end point, the assay is conceptually very similar to the in vivo radiosensitivity assay of Glucksmann, described above. The positive predictive value of this assay also supports the hypothesis that short-term damage to the entire tumor cell population may usefully reflect tumor sensitivity in vivo.

Assays Based on Changes in Cell Membrane Function

Changes in cell membrane function have usually been measured by either loss of monolayer adherence or dye exclusion. Both types of assays have produced positive clinical correlations (table 1). These assays are conceptually similar to the above-described morphology assays, except that there is a defined objective end point for cell damage and the assays are quantitative. A technical problem in the assay based on monolayer adherence is the difficulty in determining which of the adherent and nonadherent cells are tumor cells as opposed to fibroblasts.

Dye exclusion assays have long been used to quantify cell viability. Important technical problems in dye exclusion assays are as follows. (*a*) Sufficient time must elapse following drug treatment for lethally damaged cells to lose their membrane integrity. (*b*) The surviving cell fraction may continue to proliferate during the time required for the lethally damaged fraction to lose membrane integrity. (*c*) Early disintegration of some lethally damaged cells (so that they may not later be present to stain with dye) must be recognized and accounted for. (*d*) There must be a way of specifically distinguishing the tumor cells from the nontumor cells (chiefly macrophages) present in the cultures. Potential solutions to these technical problems have recently been described (38,75,104,105). Clinical correlations with dye exclusion assays have been reported from three different laboratories, and the data have compared favorably with those reported for clonogenic assays (table 1).

Virtues of dye exclusion assays include their speed and relative simplicity, plus the fact that they may require only one-tenth the number of cells required for agar cloning assays. They also detect cell kill in the nondividing cell fraction and may, accordingly, have unique advantages in new drug screening for agents capable of killing G_0 cells. Dye exclusion assays may also be valuable in the testing of neoplasms which proliferate poorly in culture. To our knowledge, a recently described dye exclusion assay is the only assay reported to date which has the capability of detecting cytotoxicity of individual cell populations in a heterogeneous culture of different normal and neoplastic cell types (104,105). We feel that further investigations into the possible clinical and drug screening applications of dye exclusion assays are clearly warranted.

Assays of Resistance Loci

Resistance loci are factors responsible for resistance of the tumor to damage produced by antitumor drugs. Examples of resistance loci are target enzymes, activation or inactivation enzymes, and drug uptake into cells (106–110). Examples of these types of tests include the following: (*a*) assays of the levels of the target enzyme aspartate transcarbamylase in tumor cells and the inhibition of this enzyme by the experimental cytostatic agent PALA (111); (*b*) assays of the levels of uridine kinase (112) and phosphoribosyl transferase (106,113), which activate 5-FU, as well as deoxycytidine kinase, which activates cytarabine (114,115); (*c*) assays of cytidine deaminase (108,115,116), which inactivates cytarabine; and (*d*) assays of methotrexate uptake into tumor cells (107).

Several clinical trials with the above types of tests have generally proved disappointing (107,110,116), although one recent correlation of 5-FU activation in patients with breast cancer was somewhat encouraging (106).

Resistance of breast cancer cells to endocrine therapy has been correlated with low levels of steroid hormone receptors (4,5). Individual breast tumors are known to be heterogeneous with respect to their estrogen receptor content in the individual cells. Additionally, the tumor cells being measured may not be tumor stem cells (70). Despite these potential pitfalls, the relative estrogen receptor content of the entire tumor correlates, in some degree, not only with responsiveness to hormonal therapy, but with prognosis independent of therapy, as measured by the disease-free interval following primary, definitive surgery. This provides further evidence that measurements taken on the entire tumor, as opposed to the subsets of cycling and clonogenic cells, may have prognostic and therapeutic validity.

Application of the resistance loci approach to other drugs and tumor systems would require knowledge of the limiting mechanisms of drug resistance, which are, in many cases, presently not known. Additionally, this approach is not easily adaptable to a generalized screening panel to individualize chemotherapy, since it would require a different type of test for each drug being screened. However, as the mechanism of resistance in human tumors becomes better understood, assays of resistance loci may yet prove to be of clinical value for specific drugs.

Assays Measuring Multiplication of the Total Number of Cells Present in the Entire Population

Total population growth assays have not been extensively studied (117). The major problem for primary human tumor cultures is that only a small minority of the tumor cells may divide in culture, thus making it difficult to detect a short-term increase in the number of cells present and correspondingly difficult to detect an inhibition of this increase. Short-term monolayer cultures were established and assays were performed on early passages in a small number of human tumors (117). Use of a continuous drug exposure did not allow accurate correlations to be made, but different drug exposure levels may have been more successful, provided that the investigators were testing tumor cells and not fibroblasts. The investigators had a relatively low success rate in establishing short-term cultures, however. A more promising type of test is based on measuring changes in the sizes of multicellular tumor spheroids (118,119). Clinical correlations have not been reported for these in vitro tests, however.

Assays Based on ^{3}H-TdR (or Similar Precursor) Incorporation

In assessing assays based on precursor incorporation, it is most important to distinguish between early labeling (done within one generation time following drug exposure) and late labeling (done at least two generation times following drug exposure) (14). Most of the clinical studies published to date have been of the early type and are accordingly subject to numerous technical pitfalls, including those relating to artifactual alterations in nucleoside intracellular pool sizes (121) and salvage versus de novo dTMP synthesis pathways (122,123). If labeling were delayed for several generation times after drug exposure, these artifacts would presumably be less pronounced and a more accurate estimate of the number of surviving proliferating cells could be obtained (fig 1; 124). An additional problem is distinguishing tumor cell labeling from nontumor cell labeling. This latter problem may be partially obviated by growing the cells on a nonadhesive substratum to prevent fibroblast growth (38,86-89). Normal cells, such as bone marrow cells and transformed lymphocytes, may potentially synthesize DNA without attachment to a culture flask, however. An alternative approach, but less convenient, is to use autoradiographic methods to allow selective identification of labeled tumor cells (38,124,125). Recently, it was reported that labeling 2-3 days (87), 4-5 days (88,89), or even longer (86) after drug exposure gave estimates of cell killing which were generally comparable to those obtained in clonogenic assays. Although, as mentioned, most published clinical studies have used the theoretically inferior early labeling technique, a number of these studies have reported impressively positive results (table 1) and variations in these labeling techniques are, fortunately, continuing to receive investigation. As was the case for cell morphology assays, nonrandomized trials based on DNA precursor labeling assays reported a superior median survival for the "treated by assay" group of patients (126), while randomized trials did not demonstrate a survival advantage (127).

Colonogenic Assays

Many different laboratories have recently reported positive clinical correlations with clonogenic assays (table 1). This is, perhaps, the strongest point in favor of these assays. We do not wish to denigrate these correlations, but it must be stated that the number of valid correlations (prospective correlations, omitting cases of mixed in vitro sensitivity in patients treated with multiple drugs) remains rather small for all assays, including clonogenic assays. Furthermore, the data do not indicate that the clinical correlations with clonogenic assays are superior to those results reported for nonclonogenic assays. As was the case for cell morphology assays and DNA precursor labeling assays, a nonrandomized trial reported a superior survival for a group of patients treated on the basis of the results of a clonogenic assay (128). There have as yet been no randomized trials reported to support the possibility that treating patients on the basis of clonogenic assay results may improve the results of chemotherapy.

In summary, neither clinical correlation data, direct experimental data, nor theoretical considerations compel us to accept the concept that clonogenic assays are more valid than nonclonogenic assays for testing the drug sensitivity of human neoplasms.

RECOMMENDATIONS FOR FUTURE WORK IN THE AREA OF PREDICTIVE CHEMOSENSITIVITY TESTING

The above data and discussion were presented to support our point of view that there has been undue emphasis placed on clonogenic assays in tumor cell biology and predictive drug sensitivity testing. This does not imply that clonogenic assays may not produce useful information in certain situations. It does imply that a more flexible attitude on the part of investigators and reviewers concerning the validity and usefulness of nonclonogenic assays could lead to important advances.

Figure 2 shows a patient with metastatic malignant melanoma. This patient has a large number of lesions which probably grew up from small embolic cell aggregates. These lesions may represent colonies in the same sense as many of the colonies seen in a typical clonogenic assay, where colonies may also grow up from small aggregates, rather than truly arising from single cells (129). When chemotherapists assess response in such a patient, they do not count the number of colonies, but instead measure the size of individual colonies and assess them for evidence of shrinkage. Thus, the clinical end point is the fate of the total tumor cell mass, which may or may not strictly reflect the result of stem cell kill.

We reviewed data from predictive in vitro assays and other theoretical concepts which suggest that *early* evidence of cell damage in the *entire* tumor cell population may predict for a multiple-log stem cell kill and also for a clinically meaningful therapeutic effect. There are other systems in which early evidence of tumor cell damage has been shown to predict for multiple-log kill of clonogenic or stem cells. These include:

(*a*) radiation therapy of cervical cancer in human patients (1,2,185,186);
(*b*) radiation therapy and chemotherapy of human tumor xenografts (137);
(*c*) chemotherapy of established cell lines in vitro (75,78);
(*d*) chemotherapy of acute leukemia (132-135) and small cell lung cancer (136,187) in human patients;
(*e*) results of chemotherapy of miscellaneous tumors in human patients compared with results of in vivo assays based on the end point of early tumor cell damage (92,102,138,139); and
(*f*) results of chemotherapy of miscellaneous tumors in human patients compared with results of in vitro assays based on the end point of early tumor cell damage (38,104,105,140-143,148).

Clonogenic cells comprise only a tiny fraction of total tumor cells (130). Cancer chemotherapy has had its greatest curative effect in neoplasms such as leukemia, lym-

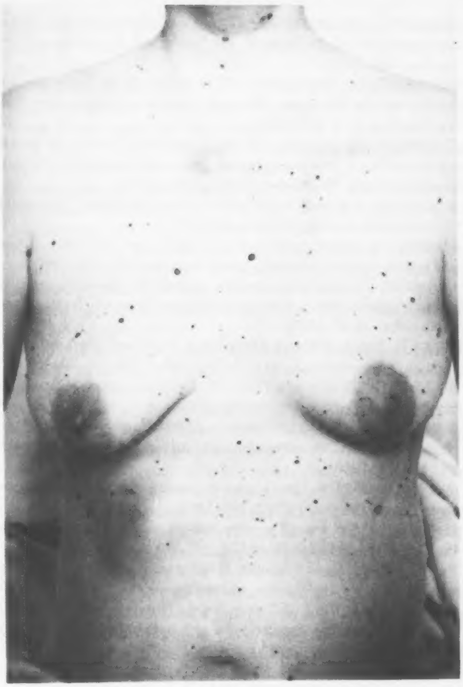

FIGURE 2.—This patient with metastatic malignant melanoma has numerous tumor colonies visible intradermally and sc. There is a huge subcutaneous colony present beneath the right costal margin and a large subcutaneous colony present in the upper right parasternal region. There are numerous smaller pigmented dermal colonies present overlying the thorax and abdomen. The colonies failed to regress, despite treatment with semustine, and the patient died a few weeks later.

phoma, testicular cancer, choriocarcinoma, small cell lung cancer, and certain pediatric solid tumors (131). These cancers share the common property of "melting away" soon after the start of effective chemotherapy. It is unlikely that this effect is mediated through the selective sterilization of a few clonogenic cells. Rather, it is likely that virtually the entire tumor cell population is being destroyed by effective chemotherapy. All of the animal tumors which are curable with chemotherapy exhibit a rapid and complete disappearance of tumor shortly following curative chemotherapy (H. Skipper, personal communication). There is growing evidence that very *early* disappearance of the *entire* leukemic blast population correlates well with eventual sustained remission (132-135). Early complete remission may also predict for prolonged survival in small cell lung cancer as well (136,187).

Kyriazis et al (137) studied the response of human tumor xenografts to chemotherapy and radiation therapy as a function of the degree of early tumor cell necrosis seen on histopathologic examination. With no early tumor cell necrosis, tumor growth was unaffected. When 5%-10% of total (not clonogenic) tumor cells underwent necrosis, there were only minor and transient responses to therapy. However, when 90% of the total tumor cells underwent necrosis, there were significant partial and complete responses. The persistence of very small numbers of morphologically viable neoplastic cells accurately predicted eventual recurrence of tumors which exhibited complete responses on gross examination. This work provides clear laboratory confirmation of the clinical results reported by Glucksmann (1) and others in predicting the radiocurability of human cervical cancer. This contrasts with the inability of clonogenic assays to predict for radiocurability (23-25) and the findings of a lack of meaningful in vivo response to chemotherapy at drug levels which produce a multiple-log clonogenic cell kill (67,68). As discussed above, the in vivo SRC assay has been shown to be of value in the prediction of responsiveness to chemotherapy (92,138,139). This assay appears to measure lysis in the entire tumor cell population as an assay end point, rather than inhibition of proliferation of clonogenic cells (99-102). In vitro assays based on the concept of early cell damage in the entire tumor cell population have correlated with clinical responsiveness to chemotherapy (38,104,105,140-143). All of these considerations provide a rational, common-sense basis for the design of an assay to measure short-term cell kill in the entire tumor cell population following drug exposure.

Which are the most promising types of assays for future study and application? We would say first and foremost that an assay must truly measure effects on tumor cells and be relatively free from artifacts. It is also of great advantage for an assay to be simple, inexpensive, rapid, and to have a high success rate. By these criteria, clonogenic assays do not appear to us to be the most promising assays for either clinical chemosensitivity testing or new drug screening. We would view clonogenic assays as being a rather clumsy method of estimating the general chemosensitivity in a proliferating cell population.

It is likely that, for some drugs or tumors, it may be more relevant to examine drug effects on mainly the proliferating cell population. In these instances, some type of ^{3}H-TdR assay would seem to us to be most practical. For reasons discussed earlier, we would favor an assay where labeling was delayed for at least 4 days following drug wash out. It is important to minimize artifacts from the labeling of normal cells. For this reason, it is important to grow cells on a nonadhesive substratum. Some investigators have plated cells in agar, but this is not more advantageous than plating cells in a liquid medium over agar (88). For maximum simplicity, we would recommend growing the cells in liquid medium in small-volume conical polypropylene tubes. Fibroblasts from primary tumor biopsies will not adhere or grow in polypropylene (104). Small-volume conical tubes would encourage cells to clump together and preserve cell-cell interactions, which may be important for normal growth. Cells may then be harvested and washed using standard semiautomated filtration units. The major caution with this approach is that lymphocyte transformation and labeling may rarely be a problem. As a quality control measure, a control culture could be processed for autoradiography to ensure that the cells being labeled were, in fact, tumor cells. In studies on established cell lines with high growth fractions, the procedure of simply counting cells and extrapolating growth curves (20) is probably the simplest and most useful method.

With some neoplasms and drugs, it may be advantageous to measure tumor cell kill in the entire (dividing and nondividing) tumor cell population. Metabolic, cell morphology, or cell adherence assays may be valid in certain situations. However, these assays either have a poor ability to distinguish between effects on tumor and nontumor cells or are subjective and poorly quantitative. The SRC assay is very promising, particularly with regard to its high assay evaluability rate and its ability to test compounds requiring in vivo activation. It is, however, expensive, particularly if a histologic end point is to be used. Additionally, it requires the testing facility to maintain a very large mouse colony. A novel dye exclusion assay system has been described which is simple, specific for tumor cells, and capable of detecting cell kill of different cell populations present in the same sample (38,104). This test can be conceived of as an in vitro counterpart of the 4-day SRC assay with a histologic end point (102). Nearly any tissue culture laboratory could easily utilize this test, and several different laboratories have already obtained promising results with this test. Many unanswered questions remain, such as what is the most appropriate time interval to wait after drug exposure before the test is performed. However, we would suggest that this assay (or a related assay based on the same principle, such as an automated assay using a flow cytometry unit) should receive intense investigation to determine for which drugs and tumors the assay is most valid and practical. We would further suggest that future comparisons of this assay with the ^{3}H-TdR assay described above would offer the greatest possibility of an early realization of the goals listed in the first paragraph of this paper.

REFERENCES

1. GLUCKSMANN A. Histological features in the local radiocurability of carcinomas. *In* The Biological and Clinical Basis of Radiosensitivity (Friedman M, ed). Springfield, United Kingdom, Thomas, 1974.
2. TROTT KR. Can tumor response be assessed from a biopsy? Br J Cancer 41:163-170, 1980.
3. BLACK MM, and SPEER FD. Further observations on the effects of cancer chemotherapeutic agents on the in vitro dehydrogenase activity of cancer tissue. JNCI 14:1147-1158, 1954.
4. JENSEN E. Hormone dependence of breast cancer. Cancer 47:2319-2326, 1981.
5. SEIBERT K, and LIPPMAN MD. Hormone receptors in breast cancer. *In* Clinics in Oncology (Baum M, ed). London, W. B. Saunders, 1982, vol 1, pp 735-794.
6. ROSENBLUM ML, DOUGHERTY DV, DEEN DF, ET AL. Potentials and limitations of a clonogenic cell assay for human brain tumors. Cancer Treat Rep 65:61-66, 1981.
7. COURTENAY FD, and MILLS J. An in vitro colony assay for human tumors grown in immune-suppressed mice and treated in vivo with cytotoxic agents. Br J Cancer 37:261-268, 1978.
8. HAMBURGER AW, and SALMON SE. Primary bioassay of human tumor stem cells. Science 197:461-463, 1977.
9. MATTOX DE, and VON HOFF DD. In vitro stem cell assay in head and neck squamous carcinoma. Am J Surg 140:527-530, 1980.
10. CHANG BK. Comparison of in vitro methods for assessing cytotoxic activity against two pancreatic adenocarcinoma cell lines. Cancer Res 43:3147-3149, 1983.
11. SELBY P, BUICK RN, and TANNOCK I. A critical appraisal of the "human tumor stem cell assay." N Engl J Med 308:129-134, 1983.
12. SELBY P, BUICK RN, and TANNOCK I. Human tumor stem cell assay. N Engl J Med 308:1479-1480, 1983.
13. VON HOFF DD, and WEISENTHAL L. In vitro methods to predict for patient response to chemotherapy. Adv Pharmacol Chemother 17:133-156, 1980.
14. WEISENTHAL LM. In vitro assays in preclinical antineoplastic drug screening. Semin Oncol 8:362-376, 1981.
15. HAMBURGER AW. Use of in vitro tests in predictive cancer chemotherapy. JNCI 66:981-988, 1981.
16. TANNEBERGER S, and NISSEN E. Predicting response of human solid tumors to chemotherapy. Cancer Treat Rev 10:203-219, 1983.
17. DENDY PP, and HILL BT, eds. Human Tumour Drug Sensitivity Testing In Vitro: Techniques and Clinical Applications. New York, Academic Press, 1983.
18. Editorial. Clonogenic assays for chemotherapeutic sensitivity of human tumors. Lancet 1:780-781, 1982.
19. LIEBER MM, and KOVACH JS. Soft agar colony formation assay for chemotherapy sensitivity testing of human solid tumors. Mayo Clin Proc 57:527-528, 1982.
20. NIAS AHW, and FOX M. Minimum clone size for estimating normal reproductive activity of cultured cells. Br J Radiol 41:468-474, 1968.
21. PUCK TT, and MARCUS PI. Action of x-rays on mammalian cells. J Exp Med 103:653-666, 1956.
22. SHOEMAKER RH, WOLPERT-DEFILIPPES MK, KERN DH, ET AL. Application of a human tumor colony forming assay to new drug screening. Cancer Res, 1985. In press.
23. PETERS LJ, HOPWOOD LE, WITHERS HR, ET AL. Predictive assays of tumor radiocurability. Cancer Treat Symp 1:67-74, 1984.
24. WEICHSELBAUM R, DAHLBERG W, LITTLE JB, ET AL. Cellular x-ray repair parameters of early passage squamous cell carcinoma lines derived from patients with known responses to radiotherapy. Br J Cancer 49:595-601, 1984.
25. FERTIL B, and MALAISE EP. Inherent cellular radiosensitivity as a basic concept for human tumor radiotherapy. Int J Radiat Oncol Biol Phys 7:621-629, 1981.
26. OGAWA M, BERGSAGEL DE, and MCCULLOCH EA. Chemotherapy of mouse myeloma: quantitative cell cultures predictive of response in vivo. Blood 41:7-15, 1973.
27. MCALLISTER RM, and REED G. Colony growth in agar of cells derived from neoplastic and non-neoplastic tissues of children. Pediatr Res 2:356-360, 1968.
28. ALTMAN A, CRUSSI F, RIERDAN W, ET AL. Growth of rhabdomyosarcoma colonies from pleural fluid. Cancer Res 35:1809-1812, 1975.
29. COURTENAY VD, SELBY PJ, SMITH IE, ET AL. Growth of human tumor cell colonies from biopsies using two soft-agar techniques. Br J Cancer 38:77-81, 1978.
30. ROPER PR, and DREWINKO B. Comparison of in vitro methods to determine drug-induced cell lethality. Cancer Res 36:2182-2188, 1976.

31. STEEL GG. Growth and survival of tumor stem cells. *In* Growth Kinetics of Tumors (Steel GG, ed). Oxford, Clarendon Press, 1977, pp 217-267.
32. SALMON SE, HAMBURGER AW, SOEHNLEN BJ, ET AL. Quantitation of differential sensitivities of human tumor stem cells to anticancer drugs. N Engl J Med 298:1321-1327, 1978.
33. VON HOFF D. "Send this patient's tumor for culture and sensitivity." N Engl J Med 308:154-155, 1983.
34. SHOEMAKER RH, WOLPERT-DEFILIPPES MK, and VENDITTI JM. Potentials and drawbacks of the human tumor stem cell assay. Behring Institute Mitteilungen 74:262-272, 1984.
35. BERTONCELLO I, BRADLEY TR, CAMPBELL JJ, ET AL. Limitations of the clonal agar assay for the assessment of primary human ovarian tumor biopsies. Br J Cancer 45:803-811, 1982.
36. SALMON SE, ed. Cloning of Human Tumor Stem Cells. New York, Alan R. Liss, 1980, p 7.
37. TAKASUGI B, YOHEM K, and MEYSKENS F. Variation in radiation survival curves based on tumor colony size. Proc Am Assoc Cancer Res 25:374, 1984.
38. WEISENTHAL LM, SHOEMAKER RH, MARSDEN JA, ET AL. In vitro chemosensitivity assay based on the concept of total tumor cell kill. Rec Results Cancer Res 94:161-173, 1984.
39. ALLEY MC, and LIEBER MM. Improved optical detection of colony enlargement and drug cytotoxicity in primary soft agar cultures of human solid tumour cells. Br J Cancer 49:225-233, 1984.
40. MEYSKENS FL, THOMSON SP, HICKIE RA, ET AL. Potential biological explanation of stimulation of colony growth in semi-solid agar by cytotoxic agents. Br J Cancer 48:863-868, 1983.
41. PATHAK MA, MATRISIAN LM, MAGUN BE, ET AL. Effect of epidermal growth factor on clonogenic growth of primary human tumor cells. Int J Cancer 30:745-750, 1982.
42. MEYSKENS FL, and SALMON SE. Modulation of clonogenic human melanoma cells by follicle-stimulating hormone, melatonin, and nerve growth factor. Br J Cancer 43:111-115, 1981.
43. CALVO F, CARNEY DN, BROWER M, ET AL. Hormone supplemented media for cloning human breast cancer: increased colony formation without alteration of chemosensitivity. Br J Cancer 48:683-688, 1983.
44. UITENDAAL MP, HUBERS HAJM, MCVIE JG, ET AL. Human tumour clonogenicity in agar is improved by cell-free ascites. Br J Cancer 48:55-59, 1983.
45. JOYCE RM, and VINCENT PC. Advantage of reduced oxygen tension in growth of human melanomas in semi-solid cultures: quantitative analysis. Br J Cancer 48:385-393, 1983.
46. TVEIT KM, ENDRESEN L, RUGSTAD HE, ET AL. Comparison of two soft-agar methods for assaying chemosensitivity of human tumours in vitro: malignant melanomas. Br J Cancer 44:539-544, 1981.
47. GUPTA V, and EBERLE R. Modulation of tumour cell colony growth in soft agar by oxygen and its mechanism. Br J Cancer 49:587-593, 1984.
48. KERN DH, CAMPBELL MA, COCHRAN AJ, ET AL. Cloning of human solid tumors in soft agar. Int J Cancer 30:725-729, 1982.
49. KARP JE, BURKE PJ, SAYLOR PL, ET AL. Correlation of proliferative and clonogenic tumor cells in multiple myeloma. Cancer Res 44:4197-4200, 1984.
50. TANNOCK I. Cell kinetics and chemotherapy: a critical review. Cancer Treat Rep 62:1117-1133, 1978.
51. BASERGA R. Multiplication and division in mammalian cells. New York, Marcel Dekker, 1976, pp 53-77.
52. WEISENTHAL LM. Human tumor stem cell assay. N Engl J Med 308:1478-1479, 1983.
53. STEEL GG. Growth and survival of tumor stem cells. *In* Growth Kinetics of Tumors (Steel GG, ed). Oxford, Clarendon Press, 1977, p 183.
54. SAUNDERS EF, and MAUER AM. Re-entry of nondividing leukemic cells into a proliferative phase in acute childhood leukemia. J Clin Invest 48:1299-1305, 1969.
55. GABUTTI V, PILERI A, TAROCCO RP, ET AL. Proliferative potential of out of cycle leukemic cells. Nature 224:375-376, 1976.
56. GAVASTO F. An outline of the objectives of the study of leukemic cell kinetics. *In* Unifying Concepts of Leukemia (Dutcher RM, and Chieco-Bianchi L, eds). Basel, Karger, 1973, pp 968-977.
57. DREWINKO B, PATACHEN M, YANG LY, ET AL. Differential killing efficacy of twenty antitumor drugs on proliferating and nonproliferating human tumor cells. Cancer Res 41:2328-2333, 1981.
58. BENNINGTON JL. Cellular kinetics of invasive squamous cell carcinoma of the human cervix. Cancer Res 29:1082-1088, 1969.
59. LIEB LM, and LISCO M. In vitro uptake of tritiated thymidine by carcinoma of the human colon. Cancer Res 26:733-740, 1966.
60. TANNOCK IF. The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumor. Br J Cancer 22:258-273, 1968.
61. DURIE BGM, and SALMON SE. Cell kinetic analysis of human tumor stem cells. *In* Cloning of Human Tumor Stem Cells (Salmon SE, ed). New York, Alan R. Liss, 1980, p 156.
62. TWENTYMAN PR. Sensitivity to 1,3-bis(2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea of the EMT6 tumor in vivo as determined by both volume response and in vitro plating assay. Cancer Res 38:2395-2400, 1978.
63. BERTRAND M, and DEEN DF. Factors influencing the recovery from potentially lethal damage (PLD) in mammalian cells in vitro and in vivo. Cancer Treat Rev 7:1-15, 1980.
64. SUDA T, SUDA J, and OGAWA M. Proliferative kinetics and differentiation of murine blast cell colonies in culture: evidence for variable G_0 periods and constant doubling rates of early pluripotent hemopoietic progenitors. J Cell Physiol 117:308-318, 1983.
65. MACKILLOP WJ, CIAMPI A, TILL JE, ET AL. A stem cell model of human tumor growth: implications for tumor cell clonogenic assays. JNCI 70:70-79, 1983.
66. MCCULLOCH EA, CURTIS JE, MESSNER HA, ET AL. The contribution of blast cell properties to outcome variation in acute myeloblastic leukemia (AML). Blood 59:601-608, 1982.
67. SKIPPER HE, and SCHABEL FM, JR. Tumor stem cell heterogeneity: implications with respect to classification of cancers by chemotherapeutic effect. Cancer Treat Rep 68:43-61, 1984.
68. STEPHENS TC, ADAMS K, and PEACOCK JH. Identification of a subpopulation of MeCCNU resistant cells in previously untreated Lewis lung tumors. Br J Cancer 50:77-83, 1984.
69. SALMON SE, ALBERTS DS, MEYSKENS FL, ET AL. Clinical correlations of in vitro drug sensitivity. *In* Cloning of Human Tumor Stem Cells (Salmon SE, ed). New York, Alan R. Liss, Inc, 1980, p 236.
70. SELBY P, BIZZARI JP, and BUICK RN. Therapeutic implications of a stem cell model for human breast cancer: a hypothesis. Cancer Treat Rep 67:659-663, 1983.
71. MOORE JV. Death of intestinal crypts and of their constituent cells after treatment by chemotherapeutic drugs. Br J Cancer 49:25-32, 1984.
72. IJIRI K, and POTTEN CS. Response of intestinal cells of differing topographical and hierarchical status to ten cytotoxic drugs and five sources of radiation. Br J Cancer 47:175-185, 1983.
73. MEISTRICH ML, FINCH M, DA CUNHA MF, ET AL. Damaging effects of fourteen chemotherapeutic drugs on mouse testis cells. Cancer Res 42:122-131, 1982.
74. TWENTYMAN PR. Experimental chemotherapy studies: intercomparison of assays. Br J Cancer 41:279-287, 1980.
75. WEISENTHAL LM, DILL PL, KURNICK NB, ET AL. Comparison of dye exclusion assays with a clonogenic assay in the determination of drug-induced cytotoxicity. Cancer Res 43:258-264, 1983.
76. GUPTA V, KRISHAN A, and ZUBROD CG. Correlation of in vitro clonogenic assay data with in vivo growth delays and cell cycle changes of a human melanoma xenograft. Cancer Res 43: 2560-2564, 1983.
77. GANAPATHI R, REITER W, and KRISHAN A. Intracellular adriamycin levels and cytotoxicity in adriamycin-sensitive and adriamycin-resistant P388 mouse leukemia cells. JNCI 68:1027-1038, 1982.
78. EHMANN UK, and WHEELER KT. Cinemicrographic determination of cell progression and division abnormalities after treatment with 1,3 bis(2-chloroethyl)-1-nitrosoruea. Eur J Cancer 15:461-473,

1979.

79. WILSON AP, FORD CHJ, NEWMAN CE, ET AL. A comparison of three assays used for the in vitro chemosensitivity testing of human tumors. Br J Cancer 49:57-63, 1984.

80. TVEIT KM, ENDRESEN L, RUGSTAD HE, ET AL. Comparison of two soft agar methods for assaying chemosensitivity of human tumors in vitro: malignant melanomas. Br J Cancer 44:539-544, 1981.

81. BHUYAN BK, LOUGHMAN BE, FRASER TJ, ET AL. Comparison of different methods of determining cell viability after exposure to cytotoxic compounds. Exp Cell Res 97:275-280, 1976.

82. YUHAS JM, TOYA RE, and PAZMINA NH. Neuraminidase and cell viability: failure to detect cytotoxic effects with dye exclusion techniques. JNCI 53:465-468, 1974.

83. RUPNIAK HT, DENNIS LY, and HILL BT. An intercomparison of in vitro assays for assessing cytotoxicity after a 24 hour exposure to anti-cancer drugs. Tumori 69:37-42, 1983.

84. ROPER PR, and DREWINKO B. Cell survival following treatment with antitumor drugs. Cancer Res 39:1428-1430, 1979.

85. WOLBERG WH. Considerations in the development of a predictive system for cancer chemotherapy. Arch Surg 102:344-347, 1971.

86. SHOEMAKER RH, IGEL HJ, MCLACHLAN SS, ET AL. Measurement of tumor colony growth and drug sensitivity in soft-agarose culture using a radioisotope method. Stem Cells 1:321-322, 1981.

87. TANIGAWA N, KERN DH, HIKASA Y, ET AL. Rapid assay for evaluating the chemosensitivity of human tumors in soft agar culture. Cancer Res 42:2159-2164, 1982.

88. FRIEDMAN HM, and GLAUBIGER DL. Assessment of in vitro drug sensitivity of human tumor cells using [^{3}H]thymidine incorporation in a modified human tumor stem cell assay. Cancer Res 42:4683-4689, 1982.

89. WEISENTHAL LM, DILL PL, MARSDEN JA, ET AL. Comparison of 3 in vitro chemosensitivity assays: dye exclusion, autoradiography, and agar cloning. Proc Am Assoc Cancer Res 23:184, 1982.

90. STEEL GG, ADAMS K, and STEPHENS TC. Clonogenic assays in the B16 melanoma: response to cyclophosphamide. Br J Cancer 36:618-624, 1977.

91. SELBY PJ, and THOMAS JM. Clonogenic cell survival curves for human melanoma xenografts using agar diffusion chamber and lung colony assays. Br J Cancer 41:150-153, 1980.

92. BOGDEN AE, and VON HOFF DD. Comparison of the human tumor cloning and subrenal capsule assays. Cancer Res 44:1087-1090, 1984.

93. KURNICK NB, COATS HA, and DE JESUS I. A new method for in vitro chemosensitivity assay: inhibition of metabolic CO_2 production. Biomed Pharmacol 37:351-353, 1983.

94. GOSALVEZ M, CANERO RG, and BLACO M. A screening for selective anticancer agent among plant respiratory inhibitors. Eur J Cancer 12:1003-1009, 1976.

95. WRIGHT JC, COBB JP, GUNPORT SL, ET AL. Further investigation of the relation between the clinical and tissue culture response to chemotherapeutic agents on human cancer. Cancer 15:284-293, 1962.

96. WHEELER TK, DERDY PP, and DAWSON A. Assessment of an in vitro screening test of cytotoxic agents in the treatment of advanced malignant disease. Oncology 30:362-376, 1974.

97. EBELING VK, and SPITZBART H. Zur Erfassung zytostatischer Effekte an Zellkulturen in vitro und deren gegunwartige Bedeutung fur eine individualisierte tumorchemotherapie des fortgeschrittenen Ovarialkarzinoms. Zentralbe Gynaekol 99:1041-1054, 1977.

98. BOGDEN AE, COBB WR, LEPAGE DJ, ET AL. Chemotherapy responsiveness of human tumors as first transplant generation xenografts in the normal mouse: six-day subrenal capsule assay. Cancer 48:10-20, 1981.

99. BOGDEN AE, GRIFFEN W, REICH SD, ET AL. Predictive testing with the subrenal capsule assay. Cancer Treat Rev 11:113-124, 1984.

100. SELTZER S, MCCORMICK KJ, PANJE WR, ET AL. Assessment of the subrenal capsule assay in the determination of chemotherapeutic sensitivities of head and neck cancers. Proc Am Assoc Cancer Res 24:319, 1983.

101. REALE F, BOGDEN A, GRIFFIN T, ET AL. The preservation of histologic morphology of human tumor explants in subrenal capsule assay. Proc Am Assoc Cancer Res 25:372, 1984.

102. LEVI FA, BLUM JP, LEMAIGRE G, ET AL. A four-day subrenal capsule assay for testing the effectiveness of anticancer drugs against human tumors. Cancer Res 44:2660-2667, 1984.

103. DUMONT P, VAN DER ESCH EP, JABRI M, ET AL. Chemosensitivity of human melanoma xenografts in immunocompetent mice and its histological evaluation. Int J Cancer 33:447-451, 1984.

104. WEISENTHAL LM, MARSDEN JA, DILL PL, ET AL. A novel dye exclusion method for testing in vitro chemosensitivity of human tumors. Cancer Res 43:749-757, 1983.

105. BIRD MC, BOSANQUET AG, and GILBY ED. In vitro determination of tumour chemosensitivity in haematological malignancies. Hematol Oncol, 1985. In press.

106. ARDALAN B, MACDONALD J, COONEY D, ET AL. The potential for clinical applications of in vitro assays predicting 5-FU sensitivity in man. Cancer Treat Rep 65:57-61, 1981.

107. BENDER RA, GIEGER WA, DRAKE JC, ET AL. In vitro correlates of clinical response to methotrexate in acute leukemia and Burkitt's lymphoma. Br J Cancer 34:484-492, 1976.

108. STEUART DD, and BURKE PJ. Cytidine deaminase and the development of resistance to arabinosyl cytosine. Nature 23:109-110, 1971.

109. HALL TC. Predictive tests in cancer. Br J Cancer 30:191-198, 1974.

110. BERTINO JR, HYRNIUK WM, and CAPRIZZI R. Prediction of methotrexate responsiveness of tumors in man. Natl Cancer Inst Monogr 34:179, 1971.

111. JOHNSON RK, SWYRD EA, and STARKY GR. Effects of *N*-(phosphonacetyl)-L-aspartate on murine tumors and normal tissues in vivo and in vitro and the relationship of sensitivity to rate of proliferation and level of aspartate transcarbamylase. Cancer Res 38:371-378, 1978.

112. REICHARD P, SKOLD O, KLEIN G, ET AL. Studies on resistance against 5-fluorouracil. I. Enzymes of the uracil pathway during development of resistance. Cancer Res 22:235-243, 1962.

113. REYES P. The synthesis of 5-fluorouridine-5-phosphate by a pyrimidine phosphoribosyl-transferase of mammalian origin. I. Some properties of the enzyme from P1534 mouse leukemia cells. Biochemistry 8:2057-2062, 1969.

114. CREASEY WA. Arabinosylcytosine. *In* Antineoplastic and Immunosuppressive Agents (Handbook of Experimental Pharmacology) part 2 (Sartorelli AC, and Johns DG, eds). New York, Springer-Verlag, 1975, vol 38, pp 232-256.

115. HARRIS AL, and GRAHAME-SMITH DG. The relationship of ara-c metabolism in vitro to therapeutic response in acute myeloid leukemia. Cancer Chemother Pharmacol 9:30-35, 1982.

116. SMYTH JF, ROBINS AB, and LEESE CL. The metabolism of cytosine arabinoside as a predictive test for clinical response to the drug in acute myeloid leukemia. Eur J Cancer 12:567-573, 1976.

117. BERRY RJ, LAING AH, and WELLS J. Fresh explant culture of human tumors in vitro and the assessment of sensitivity to cytotoxic chemotherapy. Br J Cancer 31:218-227, 1975.

118. YUHAS JM, TARLETON AE, and HARMON JG. In vitro analysis of the response of multicellular tumor spheroids exposed to chemotherapeutic agents in vitro or in vivo. Cancer Res 38:3595-3598, 1978.

119. ALLEY MC, and LIEBER MM. Assessment of growth and drug sensitivity in primary soft-agar tumor cell cultures by computerized volume analysis. Proc Am Assoc Cancer Res 25:373, 1984.

120. SCHLAG P, and FLENTJE D. Chemosensitivity testing of human neoplasms using the soft agar colony assay. Cancer Treat Rev 11:131-137, 1984.

121. NAKATA Y, and BADER JP. The uptake of nucleosides by cells in culture. II. Inhibition by 2-mercapto-I-(β-4-pyridethyl) benzimidazole. Biochem Biophys Acta 130:250-256, 1969.

122. WOLBERG WH. Response of DNA thymine synthesis in human tumor and normal tissue to 5-fluorouracil. Cancer Res 32:130-132, 1972.

123. TISMAN G, HERBERT V, and EDLIS H. Determination of therapeutic

index of drugs by in vitro sensitivity tests using human host and tumor cell suspensions. Cancer Chemother Rep 57:11-19, 1973.

124. LIVINGSTON RB, TITUS GA, and HEILBRUN LK. In vitro effects of DNA synthesis as a predictor of biological effect from chemotherapy. Cancer Res 40:2209-2212, 1980.
125. SKY-PECK HH. Effects of chemotherapy on the incorporation of 3H-thymidine into DNA of human neoplastic tissue. Natl Cancer Inst Monogr 34:197-203, 1971.
126. DENDY PP. The use of in vitro methods to predict tumour response to chemotherapy. Br J Cancer 41:195-198, 1980.
127. NISSEN E, TANNEBERGER S, PROJAN A, ET AL. Recent results of in vitro drug prediction in human tumour chemotherapy. Arch Geschwulstforsch 48:667-672, 1978.
128. ALBERTS DS, SURWIT EA, LEIGH S, ET AL. Improved survival for relapsing ovarian cancer patients using the human tumor stem cell assay to select chemotherapy. Stem Cells 1:294-295, 1982.
129. PAVELIC ZP, SLOCUM HK, and RUSTUM YM. Aggregate elimination and its effect on colony growth in soft agar. Proc ASCO 3:39, 1984.
130. SALMON SE. Development and applications of a human tumor colony assay for chemosensitivity testing. Rec Results Cancer Res 94:8-16, 1984.
131. FREI E. Cancer chemotherapy. Science 220:254-256, 1983.
132. BLUMENREICH MS, STRIFE A, and CLARKSON BD. A technique to quantify cytoreduction in the bone marrow induced by cytotoxic chemotherapy. J Clin Oncol 1:552-558, 1983.
133. CASSILETH PA, GERSON SL, BONNER H, ET AL. Identification of early relapsing patients with adult acute nonlymphocytic leukemia by bone marrow biopsy after initial induction chemotherapy. J Clin Oncol 2:107-111, 1984.
134. MASTRANGELO R, RICCARDI R, CORBO S, ET AL. A simple in vivo test for prediction of clinical response to a single drug in childhood acute leukemia. Proc ASCO 2:175, 1983.
135. HIDDEMANN W, CLARKSON BD, BUCHNER T, ET AL. Bone marrow cell count per cubic millimeter bone marrow: a new parameter for quantitating therapy-induced cytoreduction in acute leukemia. Blood 59:216-225, 1982.
136. COHEN MH, FOSSIECK BE, IHDE DC, ET AL. Chemotherapy of small cell carcinoma of the lung: results and concepts. *In* Lung Cancer: Progress in Therapeutic Research (Muggia F, and Rosencweig M, eds). New York, Raven Press, 1979, p 561.
137. KYRIAZIS AA, KYRIAZIS AP, KEREIAKES JG, ET AL. Histopathologic evaluation of response to treatment of human tumors grown in the nude mouse. Exp Cell Biol 51:83-95, 1983.
138. GRIFFEN TW, BOGDEN AE, REICH SD, ET AL. Initial clinical trials of the subrenal capsule assay as a predictor of tumor response to chemotherapy. Cancer 52:2185, 1983.
139. STRATTON JA, KUCERA PR, MICHA JP, ET AL. The subrenal capsule tumor assay as a predictor of clinical response to chemotherapy: three years of experience. Gynecol Oncol 19:336-347, 1984.
140. BOSANQUET AG, BIRD MC, PRICE WJP, ET AL. An assessment of a short-term tumour chemosensitivity assay in chronic lymphocytic leukemia. Br J Cancer 47:781-789, 1983.
141. DURKIN WJ, GHANTA VK, BALCH CM, ET AL. A methodological approach to the prediction of anticancer drug effect in humans. Cancer Res 39:402-407, 1978.
142. HOLMES HL, and LITTLE JM. Tissue culture microtest for predicting response of human cancer to chemotherapy. Lancet 2:985-987, 1974.
143. KORNBLITH PL, SMITH BH, and LEONARD LA. Response of cultured human brain tumors to nitrosoureas: correlation with clinical data. Cancer 47:255-265, 1981.
144. DIPAOLO JA. Analysis of an individual chemotherapy assay system. Natl Cancer Inst Monogr 34:240-245, 1971.
145. KONDO T. Prediction of response of tumor and host to cancer chemotherapy. Natl Cancer Inst Monogr 34:251-256, 1971.
146. WOLBERG WH, and BROWN RR. Autoradiographic studies of in vitro incorporation of uridine and thymidine by human tumor tissue. Cancer Res 22:1113-1121, 1962.
147. WILSON AP, and NOAL FE. In vitro sensitivity of human ovarian tumors to chemotherapeutic agents. Br J Cancer 44:189-200, 1981.
148. WEISENTHAL LM, LALUDE AOT, and MILLER JB. In vitro chemosensitivity of human bladder cancer. Cancer 51:1490-1496, 1983.
149. WOLBERG WH. Biochemical approaches to prediction of response in solid tumors. Natl Cancer Inst Monogr 34:189-195, 1971.
150. VOLM M, and Group for Sensitivity Testing of Tumors. In vitro short-term test to determine the resistance of human tumors to chemotherapy. Cancer 48:2127-2135, 1981.
151. HAYES FA, GREEN AA, and MAUER AM. Correlation of cell kinetic and clinical response to chemotherapy in disseminated neuroblastoma. Cancer Res 37:3766-3770, 1977.
152. RAICH PC. Prediction of therapeutic response in acute leukemia. Lancet 1:74-76, 1978.
153. WOLBERG WH. Considerations in the development of a predictive system for cancer chemotherapy. Arch Surg 102:344-347, 1971.
154. ZITTOUN R, BOUCHARD M, FACQUET-DAVIS J, ET AL. Etude de la sensibilité in vitro des cellules leucémiques aux cytotoxiques (anticytogramme). Nouv Presse Med 4:401-404, 1975.
155. MATTERN J, KAUFMANN M, WAYSS K, ET AL. Clinical correlates of in vitro effect of adriamycin in advanced lung carcinoma. Klin Wschr 54:665-670, 1976.
156. CHEN SL, SNOGA JR, and CROFT H. A short-term in vitro drug screening assay for cancer chemotherapy. Clin Chem 28:1852-1856, 1982.
157. POULSEN HS, BICHELL P, and ANDERSON J. Short-term culture of human breast cancer: in vitro effects of hormones related to patient response. Br J Cancer 46:67-74, 1982.
158. SONDAK VK, BERTELSEN CA, TANIGAWA N, ET AL. Clinical correlations with chemosensitivities measured in a rapid thymidine incorporation assay. Cancer Res 44:1725-1728, 1984.
159. CHEN SL, SNOGA JR, and CROFT H. A short-term drug screening assay for cancer chemotherapy. Clin Chem 28:1852-1856, 1982.
160. MORASCA L, ERBA E, VAGHI M, ET AL. Clinical correlates of in vitro drug sensitivities of ovarian cancer cells. Br J Cancer 48:61-68, 1983.
161. TROPE C, and SIGURDSSON K. Use of tissue culture in predictive testing of drug sensitivity in human cancer. Correlation between in vitro results and the response in vivo. Neoplasma 29:309-314, 1982.
162. DOERJER D, TEUFEL G, PFLEIDERER A, ET AL. Zwei Jahre Erfahrung mit der in vitro-testung maligner Ovarialtumoren. Arch Gynaekol 224:358-360, 1977.
163. ALBERTS DS, SALMON SE, CHEN HSG, ET AL. In vitro clonogenic assay for predicting response of ovarian cancer to chemotherapy. Lancet 2:340-342, 1980.
164. SALMON SE, ALBERTS DS, DURIE BGM, ET AL. Clinical correlations of drug sensitivity in the human tumor stem cell assay. Rec Results Cancer Res 74:300-305, 1980.
165. VON HOFF DD, CASPER H, BRADLEY E, ET AL. Association between human tumor colony-forming assay results and response of an individual patient's tumor to chemotherapy. Am J Med 70:1027-1032, 1981.
166. PARK CH, AMARE M, SAVIN MA, ET AL. Prediction of chemotherapy response in human leukemia using an in vitro chemotherapy sensitivity test on the leukemic colony-forming cells. Blood 55:595-601, 1980.
167. PREISLER HD. Prediction of response to chemotherapy in acute myelocytic leukemia. Blood 56:361-367, 1980.
168. KAISER LR, KERN DH, CAMPBELL MA, ET AL. In vitro assessment of antineoplastic therapy: new indications for thoracotomy? J Thorac Cardiovasc Surg 82:538-541, 1981.
169. LUDWIG H, and FRITZ E. Individualized chemotherapy in multiple myeloma by cytostatic drug sensitivity testing of colony-forming stem cells. Anticancer Res 1:329-334, 1981.
170. ABHAN RF, MEYSKENS FL, MOON TE, ET AL. In vitro chemosensitivities of human tumor stem cells to the phase II drug 4′-(9-acridinylamino)methanesulfon-*m*-anisidide and prospective in vivo correlations. Cancer Res 42:4495-4498, 1982.
171. MANN BD, KERN DH, GIULIANO AE, ET AL. Clinical correlations with drug sensitivities in the clonogenic assay. Arch Surg

117:33-36, 1982.

172. MEYSKENS FL, MOON TE, DANA B, ET AL. Quantitation of drug sensitivity by human metastatic colony forming units. Br J Cancer 44:787-797, 1981.

173. BALLET F, PETIT J, POUPON R, ET AL. Soft agar clonogenic assay for predicting chemosensitivity of human tumor cells from malignant effusions. Biomedicine 35:177-178, 1981.

174. VON HOFF DD, CLARK GM, STOGDIL BJ, ET AL. Prospective clinical trial of a human tumor cloning system. Cancer Res 43:1926-1931, 1983.

175. KIRKWOOD JM, and MARSH JC. In vivo drug sensitivity assay of clonogenic human melanoma cells and correlation with treatment outcome. Cancer Res 43:3434-3440, 1983.

176. KITTEN CM, VON HOFF DD, BENNETT EV, ET AL. The human tumor clonogenic assay in the treatment of patients with lung cancer. Ann Thorac Surg 36:408-410, 1983.

177. CARNEY DH, BRODER L, EDELSTEIN M, ET AL. Experimental studies of the biology of human small cell lung cancer. Cancer Treat Rep 67:27-35, 1983.

178. MATTOX DE, VON HOFF DD, CLARK GM, ET AL. Factors that influence growth of head and neck squamous carcinoma in the soft agar cloning assay. Cancer 53:1736-1740, 1984.

179. BERTELSEN CA, SONDAK VK, MANN BD, ET AL. Chemosensitivity testing of human solid tumors. Cancer 53:1240-1245, 1984.

180. HUG V, THAMES H, BLUMENSCHEIN GR, ET AL. Normalization of in vitro sensitivity testing of human tumor clonogenic cells. Cancer Res 44:923-928, 1984.

181. VON HOFF DD, HARRIS GJ, JOHNSON G, ET AL. Initial experience with the human tumor stem cell assay: potential and problems. *In* Cloning of Human Tumor Stem Cells (Salmon SE, ed). New York, Alan R. Liss, Inc, 1980, p 120.

182. HAMBURGER AW. The Salmon-Hamburger "stem" cell assay. *In* Human Tumor Drug Sensitivity Testing In Vitro (Dendy PP, and Hill BT, eds). New York, Academic Press, 1983, pp 113-119.

183. WEISENTHAL LM. Human tumor stem cell assay. N Engl J Med 308:1478-1479, 1983.

184. BUICK RN. In vitro clonogenicity of primary human tumor cells: quantitation and relationship to tumor stem cells. *In* Cloning of Human Tumor Stem Cells (Salmon SE, ed). New York, Alan R. Liss, Inc, 1980, pp 15-20.

185. DUBRAUSZKY V. Assessment of radiosensitivity of carcinoma of the cervix. J Obstet Gynecol Br Common 73:41-43, 1966.

186. DUBRAUSZKY V, and ROTTE K. Die Beurteilung der Strahlensensibilitatbeim Collumkarzinom. Strahlentherapie 141:390-395, 1971.

187. LENHARD RE, WOO KB, and ABELOFF MD. Predictive value of regression rates following chemotherapy of small cell carcinoma of the lung. Cancer Res 43:3013-3017, 1983.

188. MILLER BE, MILLER FR, and HEPPNER GH. Interactions between tumor subpopulations affecting their sensitivity to the antineoplastic agents cyclophosphamide and methotrexate. Cancer Res 41: 4378-4381, 1981.

Comparative Pharmacokinetics and Metabolism of Doxorubicin and Epirubicin in Patients With Metastatic Breast Cancer[1,2]

J. Robert,[3,*] P. Vrignaud,[3] T. Nguyen-Ngoc,[3] A. Iliadis,[4] L. Mauriac,[3] and P. Hurteloup[5,6]

We have studied the metabolism and pharmacokinetics of epirubicin (4′-epidoxorubicin), as compared to those of doxorubicin, in a total of 16 patients with metastatic breast cancer. Epirubicin metabolism markedly differs from that of doxorubicin, in that large amounts of glucuronides of epirubicinol and epirubicin were observed in plasma as well as in urine samples. The total plasma clearance of epirubicin was significantly higher than that of doxorubicin; this was interpreted as due to an increase of the distribution volume of epirubicin, since the terminal plasma half-lives of both drugs were similar. No significant modifications were observed in patients with hepatic metastases. The differences in the pharmacokinetic and metabolic properties of the two drugs may be responsible for the differences in toxicity that have been noticed between them in previous phase I and II studies. [Cancer Treat Rep 69:633–640, 1985]

Epirubicin (4′-epidoxorubicin) is a new anthracycline antibiotic which has been selected for its reduced cardiac toxicity in experimental tumor models (1,2) and in human phase I trials (3). Epirubicin is an epimer of doxorubicin, having a different configuration of the hydroxyl substituent in the C-4′ position of the sugar daunosamine. A therapeutic activity comparable to that of doxorubicin coupled with a lower acute and chronic toxicity has been reported in phase II studies (4,5).

From in vitro experiments, Plumbridge and Brown (6) have established that interactions of doxorubicin and epirubicin with DNA were similar. Moreover, Hill and Whelan (7) have emphasized the similarities of the cytotoxic and kinetic effects of doxorubicin and epirubicin in several lines of murine and human cells.

Pharmacokinetic studies have been conducted in animals (8) and humans (3,9–12). In several studies, the only metabolite of epirubicin was a 13-dihydro-reduced metabolite (epirubicinol) similar to the 13-dihydro metabolite of doxorubicin, doxorubicinol. However, in the studies of Weenen et al (12,13) and Deesen and Leyland-Jones (14), glucuronides were found in the plasma and urine samples of patients treated with epirubicin. Glucuronides of doxorubicinol aglycone had been observed before in the urine of doxorubicin-treated patients by Takanashi and Bachur (15), but they were never detected in plasma. Some differences in the pharmacokinetic parameters have also been reported. Natale et al (10) and Weenen et al (12) observed that epirubicin has a shorter terminal half-life and a higher total plasma clearance than doxorubicin.

We have carried out a pharmacokinetic and metabolism study of epirubicin as compared to doxorubicin in a total of 16 patients with metastatic breast cancer who were treated with combined chemotherapy containing either doxorubicin or epirubicin. Our results point out a definite difference in the metabolism of these compounds, and some differences in the plasma disposition kinetics after iv administration.

PATIENTS AND METHODS

Patients

The 16 patients entering the study were treated for metastatic breast carcinoma according to a phase III comparative randomized protocol. The purpose of this trial was to compare two therapeutic regimens containing

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[3] Fondation Bergonié, Bordeaux, France.

[4] Faculté de Pharmacie, Marseille, France.

[5] Farmitalia Carlo Erba, La Defense, France.

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[*] *Reprint requests to:* Dr. J. Robert, Fondation Bergonié, 180 rue de Saint-Genès, 33076 Bordeaux, Cedex, France.

either epirubicin or doxorubicin. The median age of the patients was 53. The diagnosis was assessed upon histological examinations. The metastases were documented with pulmonary radiography, liver tomodensitometry or echography, and bone scintigraphy followed by hot-spot radiography. Previous treatment included surgery in 11 cases, radiotherapy in six, and chemotherapy in four. Four patients had received no previous treatment. The extension of the disease was as follows: six patients had nodes, six had liver metastases, nine had lung metastases, and nine had bone metastases. None of these patients had signs of impaired renal or hepatic functions, and none had creatinemia $< 118\ \mu$mol. L^{-1} and bilirubinemia $< 16\ \mu$mol. L^{-1}.

The treatment combined doxorubicin or epirubicin (50 mg.m^{-2}), 5-FU (500 mg.m^{-2}), and cyclophosphamide (500 mg.m^{-2}). Only the first course of treatment was studied from a pharmacokinetic point of view. The anthracycline was always injected first; the remainder of the chemotherapy was injected 1-2 hours after the injection of the anthracycline, after the first blood samplings. The pharmacokinetic data obtained could therefore have been modulated by the associated chemotherapy.

The anthracycline, doxorubicin or epirubicin, was dissolved in 125 ml of saline and perfused in a peripheral vein of the patient as quickly as possible. The duration of the injection was exactly measured. The median duration of the drug infusion was 5 minutes. Blood samplings were made at selected times after the end of the drug infusion, generally close to 5, 10, 20, and 40 minutes, and 1, 2, 4, 8, 24, 32, and 48 hours. The blood was collected on EDTA-coated Vacutainer tubes, and was immediately centrifuged. The plasma was frozen until analysis. Urine specimens were obtained in seven patients. Cumulative excretion was not studied; only selected periods of excretion (22-24, 46-48, 70-72, and 94-96 hours) were studied, which were compared to the simultaneous excretion of creatinine. Urine samples were analyzed immediately after collection, since it was shown that anthracyclines were not stable in this fluid, even in a frozen state.

Extraction and Analysis of Anthracyclines and Their Metabolites

Plasma or urine samples were supplemented with a known amount of daunorubicin as an internal standard. The mixture was extracted by the use of C-18 Sep-pak cartridges (Waters), approximately as already described (16). Briefly, plasma samples were run through cartridges containing a bonded silica (C_{18} Sep-pak, Waters) and were eluted from these minichromatographic columns with methanol. The extract was injected in a Waters high-performance liquid chromatograph (HPLC) equipped with a microbondapak-phenyl column. The solvent was a mixture of 0.1% pH 4 ammonium formate buffer and acetonitrile (68/32, vol/vol) which was run isocratically with a flow of 3 ml.min^{-1}, approximately as described by Israel et al (17). The detection was done with a Schoffel SF970 spectrofluorometer, with an excitation wavelength of 254 nm and an emission cut-off filter at 560 nm. Quantification of the peaks was done by an automatic integrator (Waters data module) and was controlled by triangulation. This technique allows an optimal separation of anthracyclines and their metabolites and is presently widely used for anthracycline determination. The sensitivity of this technique reaches 2×10^{-9} g of compound. The identification of the peaks was obtained by comparison with authentic standards provided by Farmitalia Carlo Erba.

Identification of glucuronides was performed through glucuronidase hydrolysis of a urine extract of a patient treated with epirubicin. This extract was incubated for 1 hour at 37 °C with type VII glucuronidase (Sigma) in a pH 6,8 phosphate buffer. The re-extraction of the mixture on a Sep-pak cartridge provided a new chromatogram which could be compared to the chromatogram of the unhydrolyzed extract. For the calculations, epirubicin and its metabolites were supposed to have the same specific fluorescence per mol.

Mathematical Processing of the Data

Concerning the unchanged drug, the estimation of the pharmacokinetic parameters was based on the fitting of a compartmental model to the observed plasma concentrations. The program used had already been successfully used in a previous study (18). It takes into account the duration of the infusion, and the values are weighted as a function of the uncertainties of the measurements. The detailed method is presented in the Appendix.

Concerning the data of the metabolites, a model-independent approach was used. Only terminal half-lives and areas under concentration-time curves extrapolated to infinity were evaluated, the former by linear regression analysis on the terminal elimination phase, and the latter by the trapezoidal rule. From these values, we have computed for each metabolite the ratio of the area under the concentration-time curve to that of the parent compound. This parameter is of some interest in evaluating the ratio of the metabolic clearance of the unchanged product to the total clearance of the considered metabolite.

RESULTS

Figure 1 presents a typical chromatogram of plasma extracts obtained 1 hour after the injection of doxorubicin or epirubicin into a patient. Figures 2A and 2B present a typical time-course of plasma concentrations of either epirubicin or doxorubicin and their respective metabolites.

After the injections of doxorubicin, the only compounds which were found in plasma or urine were doxo-

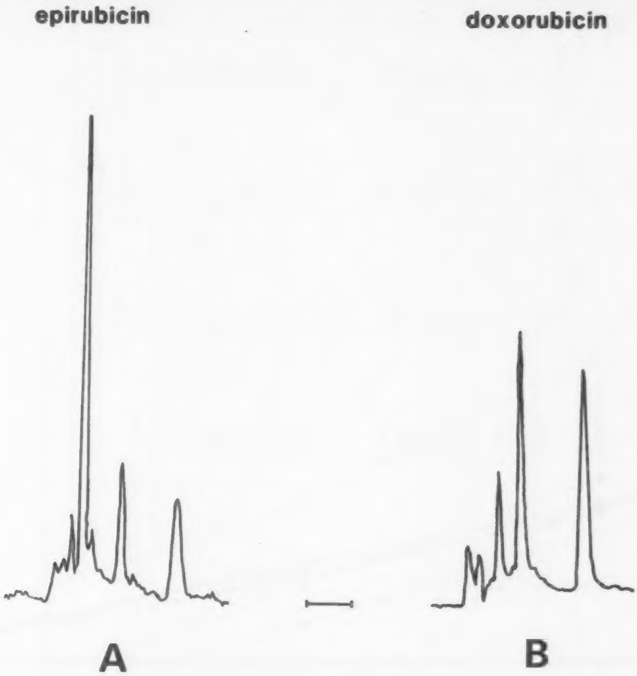

FIGURE 1.—Chromatographic separation of a plasma Sep-pak extract 1 hr after injection of 80 mg of epirubicin (A) or doxorubicin (B) in 2 patients. HPLC was performed on a microbondapak-phenyl column with formate buffer/acetonitrile (68/32) as a solvent (3 $ml.min^{-1}$). Peaks were identified as follows: (1) epirubicinol glucuronide; (2) epirubicin glucuronide; (3) epirubicinol or doxorubicinol; (4) epirubicin or doxorubicin; and (5) daunorubicin (internal standard). The bar represents 1 min.

rubicin itself and its reduced metabolite doxorubicinol. The plasma level of doxorubicinol was much lower than that of doxorubicin at the beginning of the treatment, but reached the doxorubicin level 4 hours after the injection. In contrast, epirubicin gave several metabolites: two very polar metabolites, epirubicinol, and two less polar metabolites. The two polar metabolites were identified as glucuronides of epirubicinol and epirubicin, respectively, by comparison of their retention times with those of authentic standards and by glucuronidase treatment. The position of the conjugation was not studied. The two less polar metabolites migrated as standards of doxorubicinol aglycone and doxorubicin aglycone. It must be emphasized that the two polar metabolites were quantitatively very important: epirubicin glucuronide reached a concentration higher than that of epirubicin during a long period of time. Epirubicinol reached similar concentrations as doxorubicinol after a doxorubicin injection. The two less polar compounds were observed only in some plasma samples of some patients, and, when present, reached low levels.

The kinetic parameters of the parent compounds doxorubicin and epirubicin are presented in table 1. The mean half-lives of doxorubicin and epirubicin were 20.0 and 18.3 hours, respectively. These values were not significantly different from each other. In contrast, the total plasma clearance of epirubicin (60.2 $L.hr^{-1}$) was significantly higher than that of doxorubicin (33.4 $L.hr^{-1}$). The volume of distribution was about two times higher for epirubicin than for doxorubicin.

Table 2 presents the half-lives and exposures obtained for the main metabolites of the two drugs, as the ratio of their area under the plasma curve (AUC) to that of the parent compound. The half-lives of the 13-dihydro derivatives were of the same order of magnitude as those of the parent compounds, whereas the half-lives of the glucuronides appeared to be somewhat shorter than those of epirubicin or epirubicinol. In both cases, the AUC of the 13-dihydro metabolite was about one-half that of the parent drug. Plasma levels of epirubicin glucuronide were very high, and its AUC was higher than that of epirubicin in several cases.

Urine specimens were available for 4 days in only some patients (table 3). The concentration of the parent drug

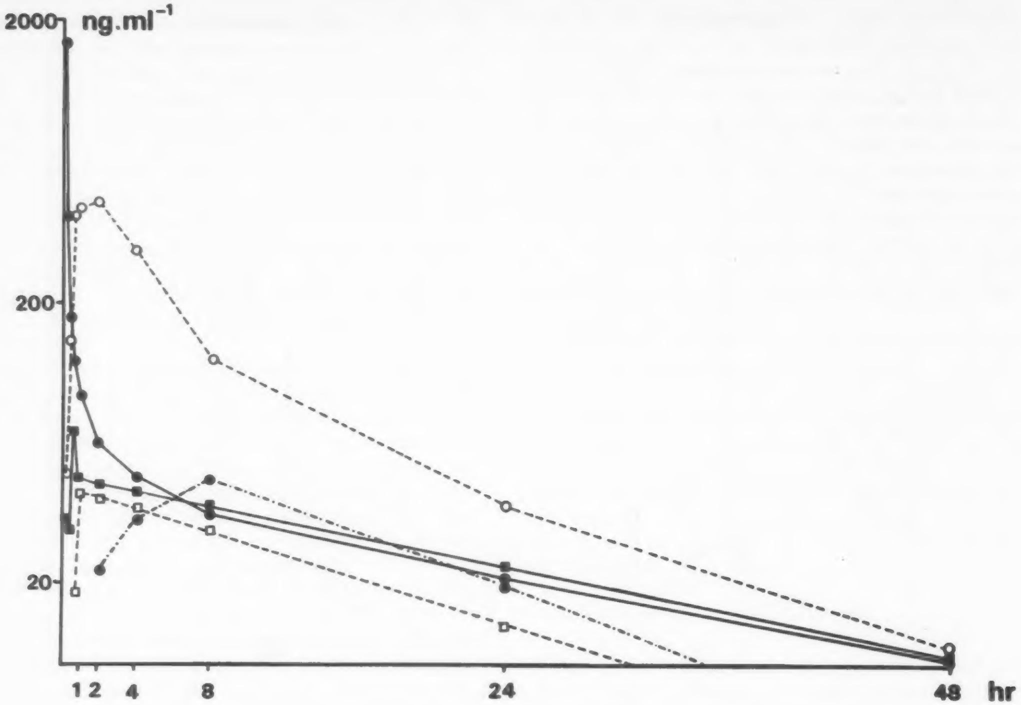

FIGURE 2A.—Time-course of the plasma concentrations of anthracyclines and their metabolites obtained in a patient treated with epirubicin. ———●——— = parent compound (epirubicin); ———■——— = 13-dihydro metabolite (epirubicinol); ---○--- = epirubicin glucuronides; ---□--- = epirubicinol glucuronides; and -·-·⊕-·-·- = doxorubicin aglycone. No doxorubicin aglycone was detected in this patient.

largely exceeded that of the metabolites for each sample. Glucuronides were found in urine, after injection of epirubicin, but aglycones were never detected. Calculation of terminal half-lives from urine excretion rates over 96 hours gave a mean value of 34.7 hours for epirubicin and a single value of 36.7 hours for doxorubicin.

The presence of hepatic metastases in three patients treated with doxorubicin and three treated with epirubicin did not modify significantly the pharmacokinetic parameters. It must be emphasized, however, that the hepatic function was not highly modified in these patients, since bilirubinemia was always < 16 μmols/L.

DISCUSSION

The existence of glucuronides in the plasma and urine of patients treated with epirubicin has been recognized first by Weenen et al (12,13) and Deesen and Leyland-Jones (14). However, these authors found much smaller plasma levels than we did. This is probably due to our original technique of extraction of anthracyclines from biological fluids (16). We have verified with authentic standards that the Sep-pak procedure gave quantitative recoveries of glucuronides with methanol as the final eluent, whereas the classical organic extraction techniques gave only minor recoveries of these polar compounds. 7-Hydroxyaglycones and 7-desoxyaglycones were also quantitatively recovered by our procedure, as already mentioned (16). The importance of the glucuronide formation may be of interest in the explanation of the differences of toxicity between doxorubicin and epirubicin, which were reported in phase I and II studies (5). It must be emphasized that doxorubicin and daunorubicin are never present as glucuronides in the plasma of patients treated with these drugs. The significance of this pathway of metabolism of epirubicin is not well understood and requires additional investigations.

We observed similar terminal half-lives for doxorubicin and epirubicin, and these half-lives were somewhat shorter than those reported by other authors for epirubicin as well as doxorubicin. This compound has been reported to have a terminal half-life ranging from 19 hours (18) to 70 hours (12). The low values that we observed in this study are probably related to the fact that we did not measure

TABLE 1.—Pharmacokinetic parameters obtained from the mathematical modeling of the levels of the parent drugs

Patient No.	Dose (mg)	α (hr^{-1})	β (hr^{-1})	γ (hr^{-1})	A ($L^{-1} \times 10^3$)	B ($L^{-1} \times 10^3$)	C ($L^{-1} \times 10^3$)	Elimination half-life (hrs)	Total plasma clearance ($L.hr^{-1}$)	Volume of distribution (L)
					Doxorubicin					
1	80	10.5	0.747	0.0363	123	1.20	0.816	19.1	27.9	484
2	80	15.9	0.711	0.0484	144	2.43	1.02	14.3	29.8	391
3	75	11.4	0.819	0.0397	125	0.961	0.848	17.4	29.9	482
4	85	10.5	0.316	0.0142	119	0.800	0.170	48.8	38.8	1281
5	85	16.4	4.015	0.0408	138	1.63	1.11	17.0	24.8	421
6	75	6.89	—	0.0448	55.3	—	0.878	15.5	36.2	575
7	75	6.60	—	0.0853	70.0	—	0.398	8.12	46.3	280
Mean ± SD								20.0 ± 12.2	33.4 ± 6.9	559 ± 307
					Epirubicin					
8	75	9.85	1.36	0.0354	112	2.32	0.889	19.6	26.8	493
9	70	6.86	—	0.0400	11.6	—	0.262	17.3	121	2416
10	80	16.9	1.03	0.0480	181	1.19	0.660	14.5	39.0	439
11	80	13.5	1.30	0.0359	108	2.47	0.605	19.3	37.9	677
12	90	9.72	0.396	0.0426	62.6	0.510	0.485	16.3	82.8	725
13	85	13.3	0.317	0.0346	40.7	0.287	0.232	20.0	93.9	1733
14	80	13.5	0.300	0.0323	135	0.600	0.243	21.4	50.6	612
15	85	16.8	0.977	0.0329	141	1.82	0.537	21.1	37.6	708
16	100	19.4	2.54	0.0466	175	1.63	0.444	14.9	52.2	558
Mean ± SD								18.3 ± 2.4	60.2 ± 29.9	929 ± 639

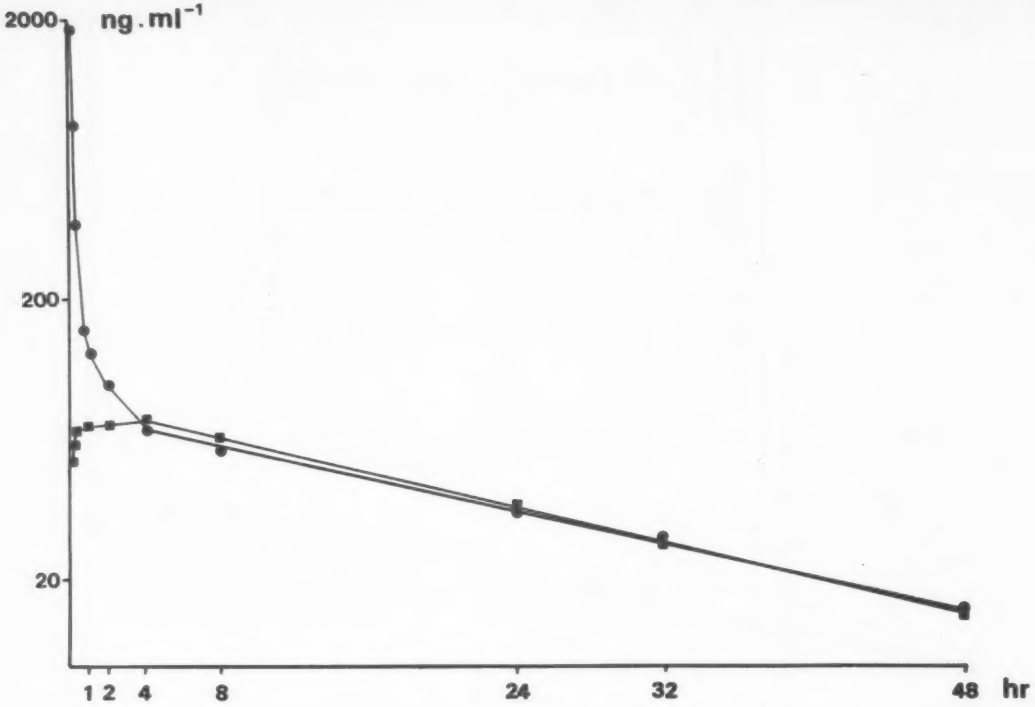

FIGURE 2B.—Time-course of the plasma concentrations of anthracyclines and their metabolites obtained in a patient treated with doxorubicin. ———●——— = parent compound (doxorubicin); and ———■——— = 13-dihydro metabolite (doxorubicinol).

TABLE 2.—Estimation of the pharmacokinetic parameters of the metabolites of doxorubicin and epirubicin*

Patient No.	13-Dihydro derivative		Epirubicin glucuronide		Epirubicinol glucuronide	
	T½	R	T½	R	T½	R
			Doxorubicin			
1	22.0	0.512				
2	16.7	0.581				
3	43.6	1.090				
4	13.3	0.433				
5	18.7	0.832				
6	14.5	0.576				
7	11.4	0.401				
Mean ± SD	20.0 ± 10.2	0.632 ± 0.228				
			Epirubicin			
8	21.8	0.197	16.1	0.467	16.1	0.148
9	—	—	16.2	0.330	—	—
10	—	—	8.80	1.703	23.8	0.510
11	33.5	0.942	8.80	1.080	10.2	0.499
12	9.56	0.976	7.14	2.478	9.16	0.676
13	17.7	0.577	14.9	1.307	—	0.239
14	20.6	0.274	10.1	0.727	—	0.183
15	23.6	0.730	12.0	2.107	13.1	0.407
16	—	—	14.5	0.650	—	—
Mean ± SD	21.1 ± 7.1	0.616 ± 0.300	12.1 ± 3.3	1.21 ± 0.71	14.5 ± 5.3	0.380 ± 0.182

*T½ = terminal half-life (hrs); R = ratio of the area under the concentration-time curve to that of the unchanged drug; dashes indicate that these parameters were not accurately evaluable.

TABLE 3.—Mean concentration of anthracyclines in urine specimens of patients treated with doxorubicin or epirubicin*

Hours	Parent compound	13-Dihydro derivative	Epirubicin glucuronide	Epirubicinol glucuronide
			Doxorubicin (2 patients)	
24	0.214	0.124		
48	0.137	0.084		
72	0.111	0.070		
96	0.056	0.042		
			Epirubicin (5 patients)	
24	0.102	0.035	0.052	0.020
48	0.048	0.018	0.021	0.009
72	0.045	0.017	0.019	0.009
96	0.029	0.010	0.013	0.005

* Results are expressed as mg of anthracyclines/mmol of creatinine.

the plasma levels after 48 hours. Another explanation could be the influence of the associated chemotherapy on terminal elimination of anthracyclines. Hartman et al (19) have shown that cyclophosphamide pretreatment modifies the disposition of doxorubicin in rats. We observed a significant difference of the total plasma clearances between doxorubicin and epirubicin. Such a difference was also reported by Natale et al (10) and Weenen et al (12). However, this difference is related to a decrease of the terminal half-life in Natale's report (10), to an increase of the distribution volume in our present work, and to both causes in Weenen's article (12). This decrease of the total drug exposure of epirubicin as compared to doxorubicin probably accounts for the limited toxicity of this drug in combination regimen studies (5). Preliminary results suggest that the important efficacy of doxorubicin is maintained by epirubicin in a combination regimen (20).

The presence of hepatic metastasis did not significantly modify the plasma disposition kinetics of the drugs in our study. When the liver function is impaired, the kinetics is modified, as demonstrated for doxorubicin by Chan et al (21) and for epirubicin by Camaggi et al (11). However, it should be noticed that among the epirubicin-treated patients, three of the four lowest clearances observed were found in liver metastasis-bearing patients.

It should be emphasized that glucuronides may not represent a preeminent way for renal excretion of epirubicin, as is usually the case for most glucuronides of drugs and hormones. The urinary concentration of glucuronides never exceeded the urinary concentration of epirubicin and epirubicinol, whereas the plasma levels of epirubicin glucuronide in the same patients usually exceeded that of epirubicin at 24 and 48 hours.

APPENDIX

The data were analyzed by fitting a two- or three-compartment model to the observed plasma concentrations, taking into account the duration T of the infusion for the total injected dose D. For these considerations, anthracycline concentration is computed at each time t and for t ⩾ T as follows:

$$y(t) = \frac{D}{T} \sum_{j=1}^{n} \frac{A_j}{\alpha_j} \left(e^{\alpha_j T} - 1\right) e^{-\alpha_j t}$$

where n may be 2 or 3 for the two- and three-compartment models, respectively.

As already described (18), we have used a weighted least-squares method to evaluate the unknown parameters A_j and α_j. This method consists in the minimization of the expression:

$$J = \sum_{i=1}^{m} W_i \left[Y_i - y(t_i)\right]^2$$

where Y_i are the experimental values, $y(t_i)$ are the values generated from the model, and W_i are the weights defined as the reciprocals of the squares of the uncertainties of the measurements Y_i (22)

$$W_i = \frac{1}{\sigma_i^2} .$$

Uncertainties σ_i at each time t_i may be considered as proportional to the observed concentrations Y_i. This weighting takes into account the reproducibility of the analytical method and it provides maximal likelihood estimates of the unknown parameters. Consequently, the expression to minimize becomes:

$$J = \frac{1}{k^2} J' = \frac{1}{k^2} \sum_{i=1}^{m} \frac{1}{Y_i^2} \left[Y_i - y(t_i) \right]^2$$

k being the proportionality constant between σ_i and Y_i. The minimization of J′ has been done by using an iterative algorithm (23) based on the nonlinear programming principle.

A two-compartment model was chosen rather than a three-compartment model whenever the values of parameters obtained from a three-compartment model were not consistent (standard deviations of the estimates higher than these estimated parameters). Estimated values of microconstants α_j and intercepts A_j allow computation of the most relevant pharmacokinetic parameters, the total plasma clearance (Cl), the terminal plasma half-life ($T_{1/2}$) of the drug, and its total volume of distribution (V_T) (24).

REFERENCES

1. CASAZZA AM. Experimental evaluation of anthracycline analogs. Cancer Treat Rep 63:835–844, 1979.
2. CASAZZA AM, DI MARCO A, BERTAZZOLI C, ET AL. Antitumor activity, toxicity and pharmacological properties of 4′epi-adriamycin. Curr Chemother 2:1257, 1978.
3. BONFANTE V, BONADONNA G, VILLANI F, ET AL. Preliminary phase I study of 4′epi-adriamycin. Cancer Treat Rep 63:915–918, 1979.
4. HURTELOUP P, CAPPELAERE P, ARMAND JP, ET AL. Phase II clinical evaluation of 4′epi-doxorubicin. Cancer Treat Rep 67:337–341, 1983.
5. GANZINA F. 4′Epi-doxorubicin, a new analogue of doxorubicin: a preliminary overview of preclinical and clinical data. Cancer Treat Rev 10:1, 1983.
6. PLUMBRIDGE TW, and BROWN JR. Studies on the mode of interaction of 4′epi-adriamycin and 4-demethoxydaunomycin with DNA. Biochem Pharmacol 27:1881, 1978.
7. HILL BT, and WHELAN RDH. A comparison of the lethal and kinetic effects of doxorubicin and 4′epi-doxorubicin *in vitro*. Tumori 68:29, 1982.
8. BROGGINI M, COLOMBO T, MARTINI A, ET AL. Studies on the comparative distribution and biliary excretion of doxorubicin and 4′epi-doxorubicin in mice and rats. Cancer Treat Rep 64:897–904, 1980.
9. MARTINI A, ISETTA AM, MORO E, ET AL. Studio farmacocinetico nell'uomo sulla 4′epi-doxorubicina in confronto a doxorubicina. Tumori 66(2):40, 1980.
10. NATALE N, BRAMBILLA M, LUCHINI S, ET AL. 4′Epidoxorubicin and doxorubicin toxicity and pharmacokinetics in cancer patients. *In* Proceedings of the 12th International Congress of Chemotherapy, Florence, Italy, July 19–24, 1981.
11. CAMAGGI CM, STROCCHI E, TAMASSIA V, ET AL. Pharmacokinetic studies of 4′epi-doxorubicin in cancer patients with normal and impaired renal function and with hepatic metastases. Cancer Treat Rep 66:1819–1824, 1982.
12. WEENEN H, LANKELMA J, PENDERS PGM, ET AL. Pharmacokinetics of 4′epidoxorubicin in man. Invest New Drugs 1:59, 1983.
13. WEENEN H, VAN MAANEN JMS, DE PLANQUE MM, ET AL. Metabolism of 4′-modified analogs of doxorubicin. Unique glucuronidation pathway for 4′-epidoxorubicin. Eur J Cancer Clin Oncol 20:919, 1984.
14. DEESEN PE, and LEYLAND-JONES B. Sensitive and specific determination of the new anthracycline analog 4′-epidoxorubicin and its metabolites by high pressure liquid chromatography. Drug Metab Dispos 12:9, 1984.
15. TAKANASHI S, and BACHUR NR. Adriamycin metabolism in man. Evidence from urinary metabolites. Drug Metab Dispos 4:79, 1976.
16. ROBERT J. Extraction of anthracyclines from biological fluids for HPLC evaluation. J Liquid Chromatogr 3:1561, 1980.
17. ISRAEL M, PEGG WJ, WILKINSON PM, ET AL. Liquid chromatographic analysis of adriamycin and metabolites in biological fluids. J Liquid Chromatogr 1:795, 1978.
18. ROBERT J, ILIADIS A, HOERNI B, ET AL. Pharmacokinetics of adriamycin in patients with breast cancer. Correlation between pharmacokinetic parameters and clinical short-term response. Eur J Cancer Clin Oncol 18:739, 1982.
19. HARTMAN N, BASSECHES PJ, and POWIS G. Effect of cyclophosphamide pretreatment on the short-term disposition and biliary excretion of adriamycin metabolites in rat. Cancer Chemother Pharmacol 10:11, 1982.
20. HAYAT M, HURTELOUP P, CHAUVERGNE J, ET AL. Phase III chemotherapy comparing FAC vs FEC in advanced breast cancer: preliminary results. *In* Proceedings of the 4th NCI-EORTC Symposium on New Drugs in Cancer Therapy, Bruxelles, December 14–17, 1983.
21. CHAN KK, CHLEBOWSKI RT, TONG M, ET AL. Clinical pharmacokinetics of adriamycin in hepatoma patients with cirrhosis. Cancer Res 40:1263, 1980.
22. WOLBERG JR. Prediction Analysis. Princeton, Van Nostrand, Inc, 1967, pp 36–39.
23. GOLDFARB D. A family of variable metric methods derived by variational means. Math Computat 24:23, 1970.
24. WAGNER JG. Fundamentals of Clinical Pharmacokinetics. Hamilton, Ohio, Drug Intelligence Publications, 1975.

Competitive Protein-Binding Assay for Trimetrexate[1]

James C. Drake,* Carmen J. Allegra, Gregory A. Curt, and Bruce A. Chabner[2]

A competitive protein-binding assay has been developed for trimetrexate (TMTX) based on the tight binding of this drug to human dihydrofolate reductase (tetrahydrofolate dehydrogenase). In this assay, TMTX competes with ^{3}H-methotrexate for binding to the enzyme. Free drug is separated from that bound to reductase by adsorption with dextran-albumin-coated charcoal. TMTX is measurable over a range from 2×10^{-9} to 5×10^{-8} M in plasma, with a coefficient of variation of < 10%. Measurements of TMTX in plasma, cerebrospinal fluid, and urine agreed closely with parallel determinations in aqueous solutions. [Cancer Treat Rep 69:641–644, 1985]

Methotrexate (MTX), an inhibitor of dihydrofolate reductase (DHFR), is widely used in the treatment of a variety of tumors. It is currently in clinical use against osteosarcoma, lymphoma, acute leukemia, breast cancer, and small cell lung cancer (1). MTX therapy is highly toxic to normal as well as tumor tissue; neurologic sequelae, renal failure, and myelosuppression are some of the unwanted side effects (1). An equally important problem is the development of resistance to MTX as the result of amplification of the target protein DHFR, an altered binding affinity of reductase, or deletion of the active transport mechanism for MTX (1). Trimetrexate (TMTX) is an antifolate specifically synthesized in an attempt to achieve an improved therapeutic index. Like MTX, TMTX is a potent inhibitor of DHFR (2). It possesses a 2,4-diamino configuration on a heterocyclic ring system, like MTX, but TMTX contains three methoxy groups on its phenylamino side group (fig 1), making it more lipophilic and thus capable of entering the cells via passive diffusion. Human leukemia cells (3) and osteosarcoma cells (4), which are resistant to MTX on the basis of impaired transport, have shown unimpaired uptake of TMTX and are sensitive to the new antifolate.

A second structural difference between TMTX and MTX may have profound effects on their relative antitumor activity and toxicity. The absence of a glutamic moiety on TMTX precludes formation of the polyglutamated form of the drug. While this change could avoid the potential toxicity associated with a depot form of the drug (5), it could also reduce efficacy since polyglutamation of MTX is believed to extend the intracellular half-life of MTX and to confer selective toxicity on those tumors which avidly form polyglutamate derivatives. These structural differences have heightened interest in TMTX as a new anticancer drug.

Assays for monitoring TMTX have been developed based on high-performance liquid chromatographic and spectrophotometric methods (6). Both assays are complex and time-consuming. We have investigated the development of a specific protein-binding assay for TMTX to allow for rapid evaluation of large numbers of clinical specimens. This assay is based on the same principle as the specific protein-binding assay developed by Myers et al (7) for MTX.

MATERIALS AND METHODS

Materials

Purified DHFR (8) obtained from MCF-7 human breast cancer cells resistant to MTX was a gift from Dr. Bernard Kaufman, National Cancer Institute, Bethesda, MD. One milliliter of this enzyme preparation formed 27.3 μmols of tetrahydrofolate/minute/mg of protein at pH 7.5 and 37°C and was able to bind 18 nmols of MTX/mg of protein at pH 7.2 and 23°C.

TMTX and unlabeled MTX were obtained from the Drug Research and Development Branch, National Cancer Institute, Bethesda, MD. Tritiated [3′,5′,9(*n*)-^{3}H] MTX (12–20 Ci/mmol) was obtained from Amersham, United Kingdom. Both tritiated and cold MTX were purified by chromatography on DEAE sephacil, Pharmacia Chemical

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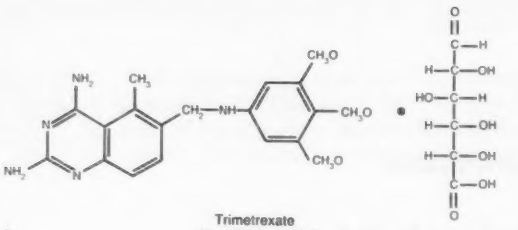
[2] Clinical Pharmacology Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

**Reprint requests to:* Dr. James C. Drake, Clinical Pharmacology Branch, Division of Cancer Treatment, National Cancer Institute, Bldg 10, Rm 6N119, National Institutes of Health, Bethesda, MD 20205.



Trimetrexate
(JB-11-glucuronate)
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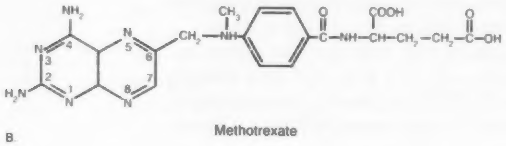

Methotrexate

FIGURE 1.—Structures of TMTX (A) and MTX (B).

Co, Uppsala, Sweden, with an NH_4HCO_3 elution gradient as previously described (9). All other chemicals were obtained from Sigma Chemical Co, St. Louis, MO.

Male mice (C57BL/10 × B10.A)F_1 were obtained from the Rodent Development Section of the National Institutes of Health, Bethesda, MD.

Competitive-Binding Assay

The assay was based on competition between ^{3}H-MTX and unlabeled TMTX for binding to DHFR. After binding was complete, the unbound drug was removed from solution by charcoal adsorption. The assay was performed at room temperature and involved the sequential addition of the following: (*a*) 4.95 nCi of ^{3}H-MTX and 50 units of heparin in 150 μl of water; (*b*) 200 μl of patient sample or standard solution of unlabeled TMTX (in water); and (*c*) 50 μl of 0.5 M potassium phosphate buffer, pH 7.2, containing 0.0027 unit of DHFR (1 unit = amount of DHFR required to bind 1 nmol of ^{3}H-MTX at 23°C and at pH 7.2) and 0.24 μmol of nicotinamide adenine dinucleotide phosphate, reduced form.

The tubes were vortex-agitated, and after 1 minute of reaction time 50 μl of a charcoal suspension was added. The stock charcoal slurry contained (in 100 ml): (*a*) Norit A-activated untreated charcoal, 10 g; (*b*) bovine serum albumin, fraction V, 2.5 g; and (*c*) high molecular weight dextran, 0.1 g. The pH of the charcoal suspension was adjusted to pH 7.2 with 1 N NaOH prior to the additions. Samples were then vortex-agitated and immediately centrifuged at 1000 × *g* for 30 minutes. A 200-μl aliquot of the supernate was withdrawn, added to 10 ml of Ready-Solv (Smith, Kline, Beckman, Philadelphia, PA), and counted in a Searle liquid scintillation counter.

A standard curve was constructed (fig 2) using precisely weighed amounts of TMTX first dissolved in methanol and then serially diluted in 0.1 M phosphate buffer, pH

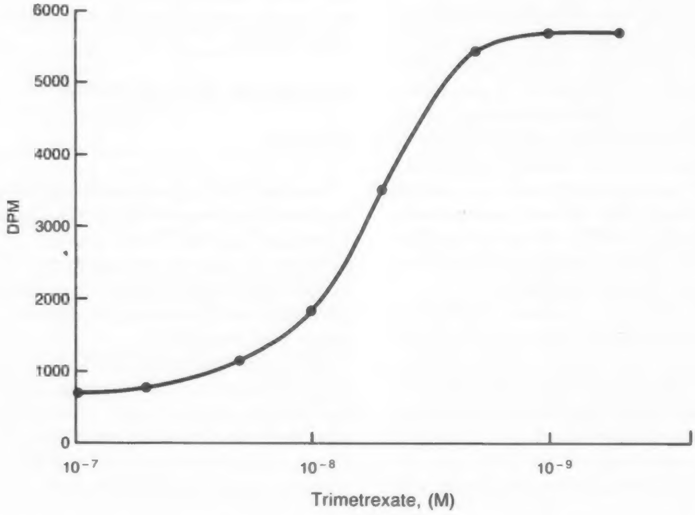

FIGURE 2.—Standard curve of TMTX concentration vs amount of ^{3}H-MTX bound to DHFR. Each sample contained 4.95 nCi of ^{3}H-MTX, 200 μl of standard TMTX solutions, 50 μl of 0.5 M potassium phosphate buffer (pH 7.2), and enzyme. Samples were processed at room temperature.

7.2. All concentrations were verified by spectral analysis using an extinction coefficient of 4550 at 330 nm in methanol (Research Triangle Institute, personal communication). It was also found that to prepare standard solutions of TMTX, it was necessary to first dissolve the compound in methanol then serially dilute it in a solution of 1 mg/ml of bovine serum albumin. Bovine serum albumin was necessary to prevent the compound from precipitating with repeated freezing and thawing.

RESULTS

Previous studies have shown that the binding of MTX to *Lactobacillus casei* DHFR is optimal at pH 6.2 (7). It was found that with the human DHFR the binding curves generated for MTX were identical at pH 6.2 and 7.2. We therefore chose to run the assay at the physiologic pH of 7.2. TMTX readily competed with MTX (fig 2). Under the assay conditions, TMTX concentrations in the range of 1-10 nM in the assay tube or $\geqslant$ 2 nM in undiluted plasma could be detected with < 10% coefficient of variation (fig 2).

To confirm the utility of the assay for measurement of drug in biological fluids, a series of standard TMTX samples were made up in plasma, urine, and cerebrospinal fluid. These were then measured and compared to standard amounts of TMTX in phosphate-buffered aqueous solution (0.05 M), pH 7.2. Values in all of the above body fluids were found to be identical to both the binding curves for TMTX in aqueous solution. Thus, plasma, urine, or cerebrospinal fluid did not interfere with the assay in amounts up to 200 μl. It was first necessary to adjust the pH of urine to 7.2 to duplicate the binding curve of TMTX in water.

To determine if the assay would work in vivo, a plasma decay curve was generated by obtaining blood samples from mice that had received 10 mg/kg ip. The results are shown in figure 3. The disappearance of TMTX from plasma exhibited first-order elimination kinetics with a half-life of 1.5 hours. The assay was sensitive to TMTX levels as low as 2.0×10^{-9} M or 0.3 pmol of TMTX/200 μl of plasma assayed. The coefficient of variation for samples in plasma, urine, or cerebrospinal fluid was found to be < 10%.

DISCUSSION

Clinical trials of TMTX are awaited with considerable interest because of the differences between this compound and MTX in terms of lipid solubility and experimental antitumor activity. TMTX lacks the terminal glutamate of MTX; instead it has a trimethoxy-substituted phenyl amine which confers high lipid solubility. This property has two conspicuous advantages: (*a*) the ability to penetrate MTX-resistant cells which lack the active transport carrier for physiologic folates and MTX (4), and (*b*) the increased ability to cross the blood-brain barrier and to enter cerebrospinal fluid. The pharmacokinetics of TMTX has not as yet been described in the human CNS, although its lipid solubility could become a major advantage in antileukemic therapy.

Second, TMTX has a spectrum of activity in preclinical testing which clearly differs from MTX. The lipid-soluble antifolate is active against the human mammary ($CD8F_1$) and colon 38 carcinoma xenografts in nude mice, while MTX is not (2). It is not known whether this activity is the result of the unique transport properties of TMTX or other factors.

Because of the clinical interest in the pharmacology of this compound, it will be important to conduct pharmacokinetic studies in parallel with early clinical trials. In particular, questions regarding the penetration into the cerebrospinal fluid and the mechanism and time course of elimination of TMTX will need to be resolved in these ini-

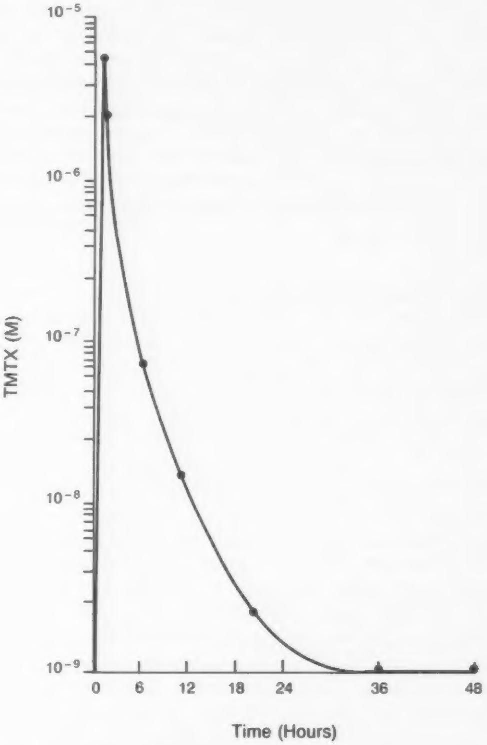

FIGURE 3.—Plasma decay curve of TMTX following ip injection of 10 mg/kg into C57BL/10 × B10.A)F_1 male mice. Blood was obtained by cardiac puncture and the TMTX levels were measured by the competitive protein-binding assay. Data were calculated from standard curve shown in fig 2. Levels at 36°C and 48°C were below the levels of detection.

tial studies. The method described herein allows for a simpler, more rapid assay of TMTX, as compared to the previously described high-performance liquid chromatographic and spectrophotometric assays (6), while preserving sensitivity. These characteristics will be useful in dealing with the numerous samples required for clinical studies. Because of the existence of a commercial assay for MTX which utilizes *L. casei* DHFR as the binding protein, we first attempted to develop an assay using *L. casei* as the enzyme source. The binding curve generated exhibited a very shallow slope with small changes in the amount of ligand bound in the TMTX concentration range of from 1×10^{-9} to 1×10^{-7} M. A steeper binding curve was obtained with human DHFR. The specificity of the competitive protein-binding assay remains to be established. It is possible that metabolites of TMTX could preserve a high degree of binding affinity for DHFR, although if this is the case, these metabolites would be active antitumor entities and would be important to measure, along with the parent compound. The metabolism of TMTX in animals is incompletely understood. Weir et al (6) have detected two urinary derivatives in small amounts. One derivative, a carbonitrile, retains modest ability to bind to DHFR (approximately 1/100,000th the affinity of TMTX). It is likely that any alteration in the diaminopteridine ring of TMTX would produce an inactive metabolite.

REFERENCES

1. Chabner BA. Methotrexate. *In* Pharmacologic Principles of Cancer Treatment (Chabner BA, ed). Philadelphia, W. B. Saunders Co, 1982, pp 229–255.
2. Bertino JR, Sawicki WL, Moroson BA, et al. 2,4-Diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]quinazoline (TMQ), a potent nonclassical folate antagonist inhibitor. I. Effect on dihydrofolate reductase and growth of rodent tumors in vitro and in vivo. Biochem Pharmacol 28:1983–1987, 1979.
3. Scanlon K, Ohnuma T, Lo RJ, et al. Effects of 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido (2,3-D) pyrimidine (BW301U) on methotrexate (MTX)-resistant human acute lymphoblastic leukemia (ALL) cell lines. Proc ASCO 23:12, 1982.
4. Diddens H, Niethammer D, and Jackson RC. Patterns of cross-resistance to the antifolate drugs trimetrexate, metoprine, homofolate, and CB 3717 in human lymphoma and osteosarcoma cells resistant to methotrexate. Cancer Res 43:5286–5292, 1983.
5. Jolivet J, Schilsky RL, Bailey BD, et al. Synthesis, retention and biological activity of methotrexate polyglutamates in cultured human breast cancer cells. J Clin Invest 70:351–360, 1982.
6. Weir EC, Cashmore AR, Dreyer RN, et al. Pharmacology and toxicity of potent “nonclassical” 2,4-diaminoquinazoline folate antagonist, trimetrexate, in normal dogs. Cancer Res 42:1696–1702, 1982.
7. Myers CE, Lippman ME, Elliot HM, et al. Competitive protein binding assay for methotrexate. Proc Natl Acad Sci USA 72:3683–3686, 1975.
8. Kaufman BT, and Kemener VF. Characterization of chicken liver dihydrofolate reductase after purification by affinity chromatography and isoelectric focusing. Arch Biochem Biophys 179:420–431, 1977.
9. Goldman ID, Lichtenstein NS, and Oliverio VT. Carrier-mediated transport of the folic acid analogue methotrexate in the L1210 leukemia cell. J Biol Chem 243:5007–5017, 1968.

Relevance of the Pharmacology of Oral Tegafur to Its Use as a 5-FU Pro-drug[1,2]

John E. Byfield,[3] Cecil L. Hornbeck,[4] Sallie S. Frankel,[3] Thomas R. Sharp,[4,*] and John C. Griffiths[5,6]

We have studied the pharmacology of iv and oral tegafur (FT) and compared the results with similar studies using continuously infused 5-FU. All patients received daily abdominal irradiation as well as FT. In eight patients receiving 1.0 g/m^2 of iv FT, serum FT levels were essentially the same as those in five patients receiving the same dose of oral FT. Oral FT appeared well-absorbed, even with abdominal irradiation. The mean serum FT achieved on a daily basis was a linear function of the oral FT dose between 1.0 and 2.5 g/m^2 and was consistently about 1000-fold higher than the resultant 5-FU level. The major FT metabolite, dehydro-FT (DHFT), was persistently present at about ten times the 5-FU level. Because of their long half-lives, both FT and DHFT accumulate during continuous therapy. When the mean serum 5-FU levels with oral FT were compared to those found during continuous 5-FU infusions, we found that oral FT was the equivalent of low-level 5-FU infusion. Oral FT at 1.0 g/m^2 was the equivalent of about 11.5 mg/kg/24 hours of 5-FU, increasing to about 17.5 mg/kg of 5-FU for oral FT at 2.5 g/m^2. The pharmacologic properties of FT appear to dictate its most useful schedule (continuous oral dosing in multiple doses) and explain why FT alone is not ideal as a 5-FU pro-drug. In addition, insight into the pharmacokinetic limitations of FT also suggests means by which its usage may be improved, including its potential application as a radiosensitizer. [Cancer Treat Rep 69:645–652, 1985]

The development of satisfactory oral forms of many cancer chemotherapeutic agents remains a largely unfulfilled goal of cancer research. This step is particularly important in the case of certain drugs like 5-FU that may be better administered under conditions of long, slow administration (1,2). The latter requires either hospitalization or semipermanent indwelling catheters (2), both of which can be expensive or cumbersome to the patient. While some drugs may readily be given orally, this is not the case for 5-FU because of its erratic intestinal absorption (3).

When the furanyl-nucleoside analog (4) of 5-FUdR, tegafur (FT), was introduced into the United States (5), it was thought that it might be used as a 5-FU pro-drug (6). Early clinical studies had shown that iv FT could be tolerated on a continuous basis (6,7) without excessive hematologic toxicity (8-11). Initial pharmacologic investigations suggested that it released, slowly and continuously, significant amounts of 5-FU (12).

Because continuously infused 5-FU is a useful clinical radiosensitizer for some tumors (13-18), we chose to study the tolerance of patients with advanced gastrointestinal tumors to the combination of oral FT and abdominal x-ray therapy (19). If oral FT produced the appropriate 5-FU pharmacokinetics, then wholly outpatient, noninterventional clinical programs exploiting 5-FU radiosensitization would be possible.

During those initial studies we noted that the 5-FU levels obtained were far below those that had been initially reported in the literature (20), an observation made simultaneously by others (21,22). We now report the more extensive data from our phase I/pharmacologic investigations with FT. The results illustrate the potential usefulness of clinical pharmacologic studies in pro-drug development and expand further on our understanding of the quantitative relationships between the pharmacology of these fluoropyrimidine agents and their clinical applications.

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[3] Department of Radiology, University of California, San Diego.

[4] Departments of Laboratory Services (*C. L. Hornbeck*) and Radiology (*T. R. Sharp*), Veterans Administration Medical Center, La Jolla, CA.

[5] Department of Pathology, University of South Carolina Medical School, Charleston.

[6] We thank Mrs. Sue Rapley for manuscript assistance.

[*] *Reprint requests to:* Dr. Thomas R. Sharp, Department of Radiology, Veterans Administration Medical Center, La Jolla, CA 92036.

MATERIALS AND METHODS

The results presented here were obtained from two separate studies. Both studies included only patients with advanced, incurable gastrointestinal cancers, and both employed a combination of abdominal irradiation and FT for the reasons cited above. The clinical details (including the radiation schedule and rationale) and results have been described elsewhere (19).

The first study was a phase I trial of iv FT and irradiation. In that trial, eight patients received a daily dose of FT administered as a single iv bolus of 1.0 g/m^2. This iv FT study was stopped when oral FT became available (23-26), and our initial experiments (reviewed here) together with the results of others (26) showed that oral FT was well-absorbed, indicating that the iv form offered no clinical advantage. However, our initial iv pharmacologic data were of use, since they permitted a direct evaluation of the effects of abdominal irradiation on FT absorption and metabolism.

The second study was similar in all important respects (type of disease, stratification, etc), differing only in that all patients received the oral preparation of the drug. Thus, each patient was given a 5-day supply of oral FT and was instructed to take each total daily dose in three equally divided doses. An overall phase I format was used, with the FT dose being escalated in sequential patient subsets to evaluate tolerance versus dose. Prior to therapy, all patients in both investigations signed an institutionally approved consent form. The tolerance of the patients to treatment with irradiation and FT has been shown in detail elsewhere (19). No evidence (*see* below) was found in either study to suggest that irradiation per se had any influence on either FT or 5-FU metabolism.

All of the oral drug was provided in tablet form (500 mg/tablet) by Mead Johnson Laboratories, while the iv formulation was provided by the National Cancer Institute. All iv FT patients (n = 8) received the drug as an iv bolus in the morning of each day of a 5-day treatment cycle. Patients in the oral FT study (n = 15) also received their drug daily during the 5 days of each treatment cycle, but the daily dose was divided into three parts, and one part was to be taken every 6-8 hours while awake (ie, three times a day). The patients were not specifically encouraged to awaken at night simply to adhere to a fixed 8-hour schedule. On each of the first 4 days of a 5-day FT cycle, each patient received one dose of 250 rads of palliative radiation to some portion of the abdomen (19).

Blood samples were taken on the second, third, and fourth mornings of each treatment cycle in both studies. These time intervals were chosen because they yielded a direct comparison of iv and oral FT constant dosing and the primary effects of cumulative radiation exposures could be followed. This sample sequence also permitted a direct comparison of the 5-FU levels generated by iv and oral FT to those found during analogous therapy using 5-FU itself (infused at 25-65 mg/kg/24 hours for 72 hours) with and without coincident radiation (27). In the iv FT study the blood sample for drug analysis was first taken and then the iv FT was injected. Samples for analysis in the oral FT study were taken only if the patient had (by history) taken the full dose of oral FT in the preceding 24 hours and there was no vomiting during that 24-hour period. Because the incidence of gastrointestinal side effects increased with the oral FT dose (19), the number of patients and samples fulfilling this criterion for serum drug analysis decreased progressively with increasing FT dose.

The preanalytic handling and processing of blood samples have been described in detail elsewhere as have our analytic techniques for determining serum levels of FT, dehydro-FT [DHFT, the major (non-5-FU) FT metabolite (cf 21)], and 5-FU (28). Due to a slight refinement in analytic technique, the lower limit for quantification for 5-FU was reduced from 25 to 10 ng/ml early in the trials so that the higher value existed during the iv trial and the lower value existed throughout the oral FT trial. Both of these values are well below both the therapeutic and toxic range for 5-day infused 5-FU (29).

RESULTS

The first comparisons of interest are the 24-hour serum levels of FT, DHFT, and 5-FU found when a daily bolus of 1.0 g/m^2 of iv FT is compared to the same dose of oral FT administered in its best tolerated schedule (30), ie, in three equally divided doses (fig 1). The levels of all three compounds (FT, DHFT, and 5-FU) were found to be comparable for the two routes of administration. The 24-hour levels of FT and DHFT were slightly lower in the iv FT group than in the oral FT group. This small difference is attributable to the different dose schedules of drug administration in the two cases vis-a-vis the time of daily phlebotomy for drug assay. The iv patients would show true residual levels having received their dose 24 hours before. The oral FT patients had received the same total dose but had taken the third fraction of the divided dose about 12 hours previously. In such a context the mean 24-hour drug level would be expected to be slightly higher with the oral schedule for a drug with a long half-life like FT (21).

However, the most impressive difference noted was in the amount of FT that persisted in the bloodstream as compared to the 5-FU level, which was barely measurable. This phenomenon is further shown in figure 2, where the blood levels of FT, DHFT, and 5-FU are plotted as a function of time over an entire treatment cycle (drug doses shown as arrows). Again, the mean serum levels of FT and DHFT tend to be slightly higher in the oral FT patients. The lower limits of assay for 5-FU are also shown in figure 2. In both of these patients receiving 1.0 g/m^2, only trace amounts of 5-FU were found in the serum. The similarity of serum FT levels irrespective of route of administration indicated that oral FT must be well-absorbed.

The relationships between the daily dose of oral FT and the mean daily serum levels of FT, DHFT, and 5-FU for the 3 assay days of each treatment cycle are shown in table 1. The oral FT dose range studied was 1.0–2.5 g/m^2. The upper dose led to excessive toxicity in all patients studied, ending the phase I aspect of the trial. In general, as the administered dose of oral FT increased, so did the mean serum FT, DHFT, and 5-FU levels. The levels of FT were approximately 1000-fold higher than 5-FU on a weight basis, while the levels of DHFT were at least tenfold higher than 5-FU.

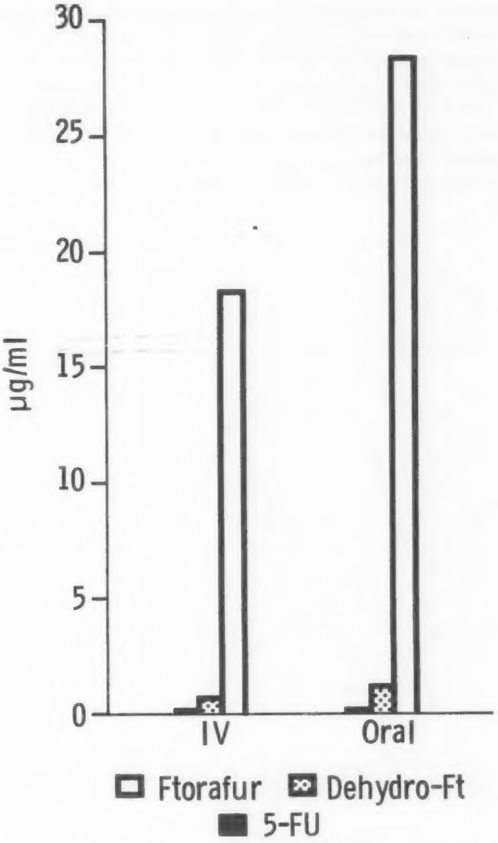

FIGURE 1.—Mean serum levels of FT, DHFT, and 5-FU in patients receiving iv or oral FT at a dose of 1.0 g/m^2/day.

The mean serum values of all the FT and DHFT levels measured in this study are plotted versus the daily FT dose in figure 3. An essentially linear relationship between the administered FT dose and the mean FT value obtained was found. The same appears true for DHFT (fig 3; table 1), although the small number of samples in the outlying points makes this conclusion less certain. For most FT levels there was a clear linear relationship with the amount of DHFT formed. This is shown in figure 4, where the mean FT levels for each oral daily dose have been plotted against the mean DHFT found in the same sample subset (again subject to greater variation at the highest FT doses, where the sample number is smallest).

There was a clear-cut tendency for both FT and DHFT to increase (ie, accumulate) on a daily basis at all doses studied (fig 5). This is to be expected for a drug with a long half-life such as FT, whose measured half-life varies from 6 to 16 hours (31). From the 24-hour residual FT levels found in the current study (table 1; fig 5), it can be estimated that the half-life of FT elimi-

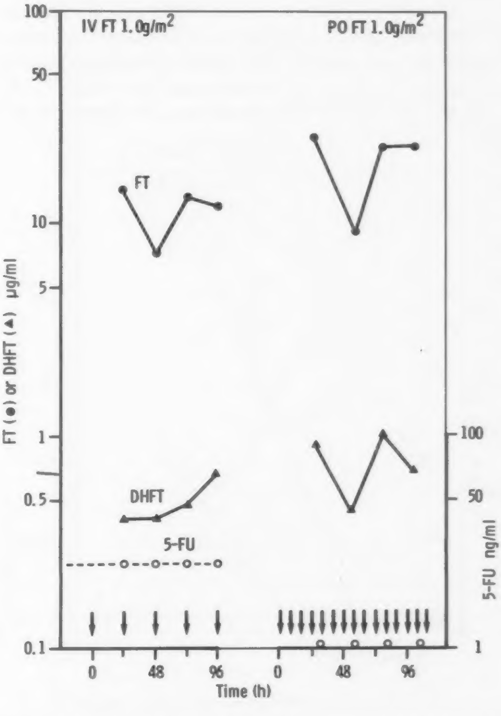

FIGURE 2.—Levels of FT and DHFT in 2 representative patients receiving iv or oral FT over 4 days. Arrows indicate times that bolus iv FT (left panel) or oral FT (right panel, 3 divided doses) was given. -O- indicates lower limits of sensitivity for 5-FU in these particular assays (*see* text).

nation in our patients had a mean value of about 11 hours.

The mean serum levels of 5-FU found are plotted as a function of the administered oral FT dose in figure 6. 5-FU levels generated during continuous infusions of 5-FU at dose rates of 25–40 mg/kg/24 hours are included for comparison. It can be seen that the 5-FU levels resulting from FT administration at doses of 1.0–2.5 g/m^2 uniformly fall *below* the 5-FU levels found with even the lowest 5-FU infusions we have studied (25 mg/kg/24 hours). Nevertheless, the quantitative relationship between the dose of administered FT and the resultant mean serum 5-FU values appears the same. Thus, the slope of the curve relating administered oral FT dose to resultant mean serum 5-FU levels has the same slope as the curve relating iv administered 5-FU to mean serum 5-FU levels (27). For all intents the oral FT data fill in the lower portions of the 5-FU dose/concentration curve. Thus, oral FT results in serum 5-FU levels equivalent to very low dose continuous 5-FU infusions.

DISCUSSION

The goal of this study was to determine the exact relationship between the dose of FT administered and the consequent serum concentrations of the released 5-FU. At the time we started our studies, the degree of 5-FU released by FT into the systemic circulation was controversial (12,21,32,33). Our specific need was for a pro-drug that released 5-FU in a consistent, *sustained*, predictable fashion. This was an absolute requirement because only sustained (and cytotoxic) levels of 5-FU are capable of radiosensitizing human tumors (34,35).

The serum FT levels we found after oral dosing are essentially the same as with iv drug, confirming the observations of Diasio et al (26) and Anttila et al (31) in studies of oral FT alone (ie, without irradiation). Therefore, FT seems to be well-absorbed and its absorption is not significantly impaired by coincident irradiation. Oral FT appears satisfactory in this regard and would be an almost ideal oral 5-FU pro-drug were it not for other factors.

The first potentially serious problem with FT relates to the nature and properties of its degradation products. These are largely DHFT (21) and 5-FU, although several other minor products have also been identified in human serum and urine (21,36). At all times studied in our investigation, we found the concentration of DHFT to be substantially higher than the serum 5-FU levels, a phenomenon also noted by others for iv FT (21). The DHFT levels shown and the tendency of DHFT to accumulate with serial daily FT administration (table 1) indicate that the half-life of this compound must be greater than or equal to FT, ie, of the order of ≥ 8 hours. DHFT is known to be more lipophil-

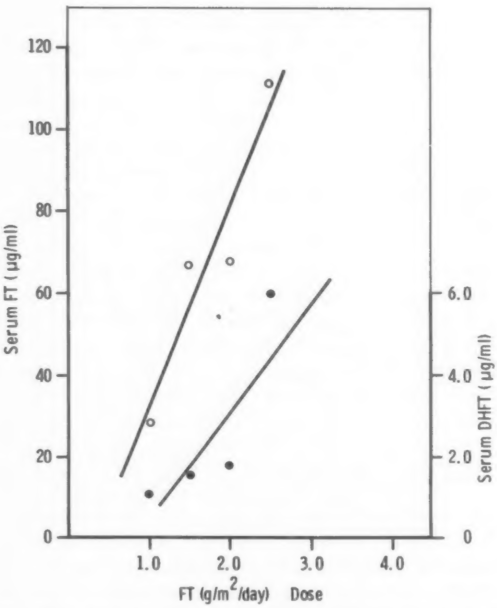

FIGURE 3.—Mean serum concentrations of FT (left ordinate) and DHFT (right ordinate) as a function of daily oral FT dose.

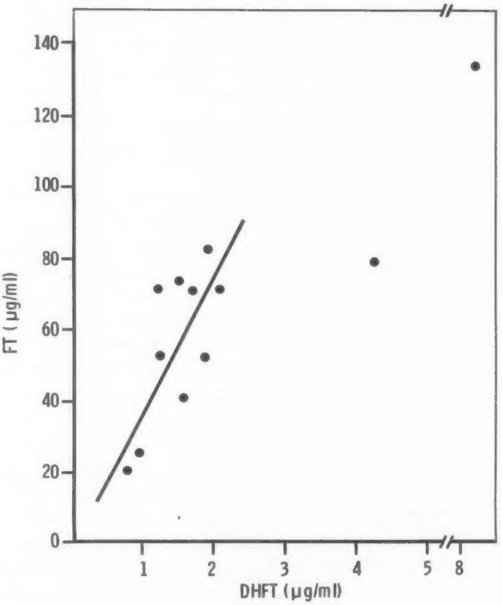

FIGURE 4.—Relationship between mean serum levels of FT and DHFT generated from various doses of oral FT.

ic than FT (or 5-FU) and may well contribute to the CNS toxicity of FT as has been suggested by Au et al (21). The tendency of both FT and DHFT to persist and accumulate may also contribute to the cumulative toxicity that limits the *total* administrable dose of even low-dose (1 g/m^2) oral FT (30).

In general, the presence of significant quantities of any intermediate product of pro-drug metabolism is unlikely to be useful and likely to be troublesome. The only exception to this would be a nontoxic metabolic intermediate necessary for the ultimate generation of the desired product. Neither the levels nor the half-life of DHFT suggests that it is a major source of 5-FU in FT-treated patients, although it has been shown to form 5-FU on decomposition (21). Existing data show that most administered FT is eventually degraded to 5-FU decomposition products (37). If DHFT were a major source of 5-FU in FT-treated patients (ie, FT → DHFT → 5-FU), one would expect blood levels of DHFT to be much higher, ie, closer to the levels of FT itself, which should be correspondingly lower. Instead, the data (table 1; figs 1 and 2) suggest that about one molecule of DHFT is formed per 20 molecules of FT. Accordingly, the formation of DHFT is probably only an undesirable side effect of FT therapy (38).

However, the major limitation of FT as a 5-FU prodrug appears to be the low levels of serum (extracellular) 5-FU formed during treatment. It is important to recognize that this is an inevitable consequence of the disparate half-lives of the two compounds. There is general agreement (12,21,31,32,39) that the half-life of FT in man, although variable, is usually about ≥ 8 hours. This must result in drug accumulation as is shown here (table 1; fig 5). In contrast, the half-life of 5-FU is around 10–15 minutes (3). Thus, most of the 5-FU formed from FT will be rapidly metabolized. Increasing the FT dose, even to very high levels, as by iv bolus, will not result in much improvement, since the major blood component will simply be the long-lived parent compound and any of its long-lived products (DHFT), rather than the desired short-lived product (5-FU). The clinical effect is therefore to substitute the side effects of the long-lived compounds for those of the desired product drug (40).

The problems are rendered even more complex in the case of a drug like 5-FU whose clearance is known to be strikingly nonlinear (3,27,41,42). At very low dose 5-FU infusions, the clearance of 5-FU is essentially that of the cardiac output, indicating that most of the drug is metabolized in extrahepatic tissues including tumor sites (27). We have proposed that this extrahepatic clearance is the major source of both the toxicity and tumor response to *infused* 5-FU, perhaps through its incorporation into RNA (27). Alternative interpreta-

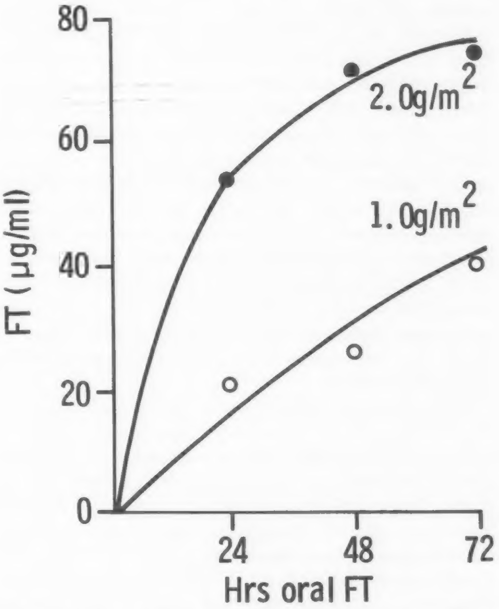

FIGURE 5.—Accumulation of serum FT during continuous oral therapy.

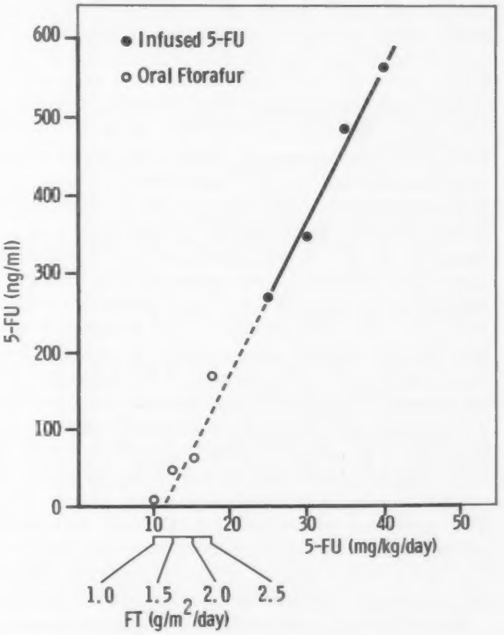

FIGURE 6.—Relationship between mean serum 5-FU levels found as a function of oral FT dose (this study) and various doses of 5-day-infused 5-FU (ref 27).

TABLE 1.—Daily mean serum levels of FT, DHFT, and 5-FU following various oral FT doses*

FT dose (g/m^2)	Day 2	Day 3	Day 4
1.0			
No. of patients	12	7	8
FT	21.3 ± 10.2	25.1 ± 18.7	41.2 ± 1.6
DHFT	0.81 ± 0.34	0.96 ± 0.46	1.60 ± 0.8
5-FU	3.36 ± 6.45	9.71 ± 10.1	13.0 ± 21
1.5			
No. of patients	11	9	8
FT	52.9 ± 22.5	70.9 ± 27.3	83.1 ± 39
DHFT	1.22 ± 0.85	1.72 ± 1.0	1.93 ± 1.1
5-FU	40.6 ± 21.8	47.3 ± 26.2	69.6 ± 32
2.0			
No. of patients	7	10	7
FT	53.4 ± 19.7	72.2 ± 27.5	74.6 ± 22.4
DHFT	0.86 ± 0.42	2.08 ± 2.2	1.5 ± 0.7
5-FU	51.0 ± 14.8	71.1 ± 20.0	73.0 ± 40
2.5			
No. of patients	1	1	3
FT	79.0	72.0	134.3 ± 57
DHFT	4.5	1.2	8.23 ± 12.1
5-FU	239	69	273 ± 285

*Levels of FT and DHFT are reported in μg/ml. Levels of 5-FU are reported in ng/ml. Values = ± SD.

tions may also be considered (41). FT, being therapeutically active only through released 5-FU, is subject to these same considerations and would therefore be optimally active when its administration schedule most closely reproduces an *effective* infusional 5-FU program.

The impact of clearance effects on serum 5-FU levels is shown in figure 6. It is only after the rate of a 5-FU continuous infusion exceeds at least 10 mg/kg/24 hours that consistent serum 5-FU blood levels can begin to be demonstrated (27). This is shown by the back extrapolation point of the infused 5-FU data curve shown in figure 6. It may be seen that a minimum infusional dose rate (slightly >10 mg/kg/day or about 0.5 mg/minute) is required to produce definable serum 5-FU levels. The data in table 1 and in figure 6 show that FT (at tolerated doses) generates levels of 5-FU equivalent to very slow rates of 5-FU infusion. Thus, an optimal "continuous-administration" dose of FT of 1.0 g/m^2/day (30) is equivalent to a 5-FU infusion rate of about 11.5 mg/kg/day. The maximally tolerated FT (11,19,40) daily dose of FT (2.5 g/m^2/day by any route) produces serum 5-FU levels equivalent to only about 17.5 mg/kg/day of infusional 5-FU. Such 5-FU levels are below those needed to induce either significant normal tissue (5-FU) toxicity or tumor responses when 5-FU is infused on a tolerable schedule over ≤ 5 days (1).

Viewed in this light the pharmacologic data appear to dictate the possible useful schedules for FT. When FT is given iv, no scheduling variation other than the low-dose "semicontinuous" schedules (ie, those originally devised outside the United States) have produced a significant response rate (4,6). When the iv dose is raised much above these modest levels, CNS toxicity intervenes (40), presumably mediated by FT itself and probably aggravated by DHFT, and the response rate actually falls (43).

Oral FT, being well-absorbed, has the same limitations but has *slightly* less CNS toxicity (30), presumably because the peak FT level for equivalent schedules is much lower (31). However, oral FT still does not produce sufficient serum 5-FU to produce clinical 5-FU toxicity or responses until it is also given on a maximally tolerated *continuous* dose basis (30). Such a schedule is realistic only with the oral route, which readily permits both multiple continuous doses (without injections) and fractionated daily doses (both of which act to reduce the peak FT levels that presumably cause acute CNS toxicity). Continuous oral FT dosing therefore comes closest to reproducing the pharmacokinetics of the true continuous 5-FU infusions recently studied by Lokich et al (2).

In summary, we believe these and related data show that FT is, in fact, a useful 5-FU pro-drug but one with significant limitations. These limitations are directly predictable from the pharmacokinetic properties of the parent compound and its catabolic products. It seems likely that only the continuous, fractionated daily oral schedule can give a meaningful idea as to the true therapeutic potential of FT. Other schedules, most particularly iv schedules (whether alone or in combination), have probably significantly underestimated its potential maximum response rate.

The primary limitation of FT lies in its failure to produce *sustained* serum 5-FU levels. This in turn is an *inevitable* consequence of the long half-life of the parent FT and the short half-life of the desired product (5-FU). This conclusion then underlines the potential importance of means by which 5-FU catabolism could be slowed. For example, Fujii et al (44) showed that the coincident administration of FT and uracil enhanced tumor accumulation of 5-FU in rodents. We have recently found evidence for a similar effect in human tumors growing in athymic mice (45). These effects are presumably mediated in part by the known capacity of pyrimidines to prolong the half-life of 5-FU through competition for catabolic pathways (46,47). Interestingly, the addition of uracil to FT therapy (in animals) has recently been shown to reduce the incidence and degree of neurotoxicity and cardiotoxicity induced by FT (48). These latter observations would support the hypothesis that the toxicity of FT can be (usefully) dissociated from that of its desired product, 5-FU (49).

We believe that this type of analysis underlines the value of pharmacologic studies in identifying specific areas for investigation, especially for agents being considered as pro-drugs for compounds of demonstrated value. Ideally, the hypothesized optimal concentration × time parameters of the primary agent should *first* be identified. A putative pro-drug may then be synthesized but it should be confirmed to produce the desired pharmacologic properties in vivo prior to extensive schedule and phase III testing. Moreover, it must be capable of doing this within the framework of an acceptable toxicity spectrum throughout its catabolic pathways. When these factors are identified in advance, the development and application of suitable prodrugs will likely be more rapid.

REFERENCES

1. SEIFERT P, BAKER LH, REED ML, ET AL. Comparison of continuously infused 5-fluorouracil with bolus injection in treatment of patients with colorectal adeno-carcinoma. Cancer 36:123-128, 1975.
2. LOKICH J, BOTHE A, FINE N, ET AL. Phase I study of protracted venous infusion of 5-fluorouracil. Cancer 48:2565-2568, 1981.
3. MYERS C. The pharmacology of the fluoropyrimidines. Pharmacol Rev 33:1-15, 1981.
4. HILLER SA, ZHUK RA, and LIDAK MY. Analogs of pyrimidine nucleosides. I. N′-(alpha-tetrahydrofuryl) derivatives of natural pyrimidine bases and their antimetabolites. Dokl Akad Nauk SSSR 176:332-337, 1967.
5. SMART CR, TOWNSEND LB, RUSHO WJ, ET AL. Phase I study of ftorafur, an analogue of 5-fluorouracil. Cancer 36:103-106, 1975.
6. FRIEDMAN MA, and IGNOFFO RJ. A review of the United States clinical experience of the fluoropyrimidine, ftorafur (NSC-148958). Cancer Treat Rev 7:205-213, 1980.
7. SMOLYANSKAYA AZ, and TRIGARINOV OA. The biological activity of antitumor antimetabolite "ftorafur." Neoplasma 19:341-345, 1972.
8. JOHNSON RK, GARIBJANIAN BT, HOUCHENS DP, ET AL. Comparison of 5-fluorouracil and ftorafur. I. Quantitative and qualitative differences in toxicity to mice. Cancer Treat Rep 60:1335-1345, 1976.
9. GARIBJANIAN BT, JOHNSON RK, KLINE I, ET AL. Comparison of 5-fluorouracil and ftorafur. II. Therapeutic response and development of resistance in murine tumors. Cancer Treat Rep 60:1347-1361, 1976.
10. BLOKHINA NG, VOZNY EK, and GARIN AM. Results of treatment of malignant tumors with ftorafur. Cancer 30:390-392, 1972.
11. VALDIVIESO M, BODEY GP, GOTTLIEB JA, ET AL. Clinical evaluation of ftorafur (pyrimidine-deoxyribose N1-2′-furanidyl-5-fluorouracil). Cancer Res 36:1821-1824, 1976.
12. BENVENUTO JA, LU K, HALL SW, ET AL. Disposition and metabolism of 1-(tetrahydro-2-furanyl)-5-fluorouracil (ftorafur) in humans. Cancer Res 38:3867-3870, 1978.
13. BYFIELD JE, BARONE RM, MENDELSOHN J, ET AL. Infusional 5-fluorouracil and x-ray therapy for non-resectable esophageal cancer. Cancer 45:703-708, 1980.
14. BYFIELD JE, BARONE RM, SHARP TR, ET AL. Conservative management without alkylating agents of squamous cell anal cancer using cyclical 5-FU alone and x-ray therapy. Cancer Treat Rep 67:709-712, 1983.
15. BYFIELD JE, SHARP TR, TANG S, ET AL. Phase I and II trial of 5-day infused 5-fluorouracil and radiation in advanced cancer of the head and neck. J Clin Oncol 2:406-413, 1984.
16. CUMMINGS BJ, RIDER WR, HARWOOD AR, ET AL. Combined radical radiation therapy and chemotherapy for primary squamous cell carcinoma of the anal canal. Cancer Treat Rep 66:489-492, 1982.
17. THOMAS G, DEMBO A, BEALE F, ET AL. Concurrent radiation, mitomycin C, and 5-fluorouracil in poor prognosis carcinoma of the cervix: preliminary results of a phase I-II study. Int J Radiat Oncol Biol Phys 10:1785-1790, 1984.
18. KEANE TJ, HARWOOD AR, RIDER WD, ET AL. Concomitant radiation and chemotherapy for squamous cell carcinoma of the esophagus. Int J Radiat Oncol Biol Phys 10:89, 1984.
19. BYFIELD JE, SHARP TR, HORNBECK CL, ET AL. Phase I study of oral ftorafur and x-ray therapy in advanced gastrointestinal cancer. Int J Radiat Oncol Biol Phys (in press).
20. HORNBECK CL, GRIFFITHS JC, FLOYD RA, ET AL. Serum concentrations of 5-fluorouracil, ftorafur, and a major serum metabolite following ftorafur chemotherapy. Cancer Treat Rep 65:69-72, 1981.
21. AU JL, WU AT, FRIEDMAN MA, ET AL. Pharmacokinetics and metabolism of ftorafur in man. Cancer Treat Rep 63:343-350, 1979.
22. AU JL, and SADEE W. 5-Fluorouracil concentrations in human plasma following *R,S*-1-(tetrahydro-2-furanyl)-5-fluorouracil (ftorafur) administration. Cancer Res 39:4289-4290, 1979.
23. ANSFIELD FJ, KALLAS G, and SINGSON J. Phase I-II studies of oral tegafur (ftorafur). J Clin Oncol 1:107-110, 1983.
24. MORGAN LR, BROWDER H, and CARTER RD. Oral ftorafur: a feasibility study. Proc Am Assoc Cancer Res and ASCO 20:397, 1979.
25. WEETH JB. Ftorafur: oral tablet trial follows intravenous phase I study. Proc Am Assoc Cancer Res and ASCO 20:307, 1979.
26. DIASIO RB, HUNTER HL, LA BUDDE JA, ET AL. Pharmacologic study of oral ftorafur: potential for improved oral delivery of 5-fluorouracil. Proc Am Assoc Cancer Res and ASCO 20:401, 1979.
27. BYFIELD JE, FRANKEL SS, HORNBECK CL, ET AL. Phase I and pharmacologic study of 72 hour infused 5-fluorouracil and hyperfractionated cyclical radiation. Int J Radiat Oncol Biol Phys. In press.
28. HORNBECK CL, FLOYD RA, GRIFFITHS JC, ET AL. Improved liquid chromatographic assay for serum fluorouracil concentrations in the presence of ftorafur. J Pharm Sci 70:1163-1166, 1981.
29. BYFIELD JE, FRANKEL SS, HORNBECK CL, ET AL. Relationships between serum 5-FU levels and toxicity. Proc ASCO 2:44, 1983.
30. BEDIKIAN AY, BODEY GP, VALDIVIESO M, ET AL. Phase I evaluation of oral tegafur. Cancer Treat Rep 67:81-84, 1983.
31. ANTTILA MI, SOTANIEMI EA, KAIRALUOMA MI, ET AL. Pharmacokinetics of ftorafur after intravenous and oral administration. Can-

cer Chemother Pharmacol 10:150-153, 1983.

32. LOO TL, BENJAMIN RS, LU K, ET AL. Metabolism and disposition of Baker's antifolate (NSC-139105), ftorafur (NSC-148958), and dichlorallyl lawsone (NSC-126771) in man. Drug Metab Rev 8:137-150, 1978.
33. BENVENUTO JA, LU K, and LOO TL. High-pressure liquid chromatographic analysis of ftorafur and its metabolites in biological fluids. J Chromatogr 134:219-222, 1977.
34. BYFIELD JE, CALABRO-JONES PM, KLISAK I, ET AL. Pharmacologic requirements for obtaining sensitization of human tumor cells in vitro to combined 5-fluorouracil or ftorafur and x-rays. Int J Radiat Oncol Biol Phys 8:1928-1933, 1982.
35. NAKAJIMA Y, MIYAMOTO T, TANABE M, ET AL. Enhancement of mammalian cell killing by 5-fluorouracil in combination with x-rays. Cancer Res 39:3763-3767, 1979.
36. BENVENUTO JA, LIEHR JG, WINKLER T, ET AL. Human urinary metabolites of 1-(tetrahydro-2-furanyl)-5-fluorouracil. Cancer Res 39:3199-3201, 1979.
37. TOIDE H, AKIYOSHI H, MINATO Y, ET AL. Comparative studies on the metabolism of 2-(tetrahydrofuryl)-5-fluorouracil and 5-fluorouracil. Gan 68:553-560, 1977.
38. EL SAYED YM, and SADÉE W. Metabolic activation of *R,S*-1-(tetrahydro-2-furanyl)-5-fluorouracil (ftorafur) to 5-fluorouracil by soluble enzymes. Cancer Res 43:4039-4044, 1983.
39. HILLS EB, GODEFROI VC, O'LEARY IA, ET AL. GLC determination for ftorafur in biological fluids. J Pharm Sci 66:1497-1499, 1977.
40. HALL SW, VALDIVIESO M, and BENJAMIN RS. Intermittent high single-dose ftorafur: phase I clinical trial with a pharmacologic-toxicity correlation. Cancer Treat Rep 61:1495-1498, 1977.
41. COLLINS JM, DEDRICK RL, KING FG, ET AL. Non-linear pharmacokinetic models of 5-fluorouracil in man: intravenous and intraperitoneal routes. Clin Pharmacol Ther 28:235-246, 1980.
42. FLOYD RA, HORNBECK CL, BYFIELD JE, ET AL. Clearance of continuously infused 5-fluorouracil in adults having lung or gastrointestinal carcinoma with or without hepatic metastases. Drug Intell Clin Pharm 16:665-667, 1982.
43. BUROKER T, PADILLA F, GROPPE C, ET AL. Phase II evaluation of ftorafur in previously untreated colorectal cancer. Cancer 44:48-51, 1979.
44. FUJII S, KITANO S, IKENAKA K, ET AL. Effect of coadministration of uracil or cytosine on the anti-tumor activity of clinical doses of 1-(2-tetrahydrofuryl)-5-fluorouracil and level of 5-fluorouracil in rodents. Gan 70:209-214, 1979.
45. TANG S, HORNBECK CL, and BYFIELD JE. Enhanced accumulation of 5-fluorouracil in human tumors in athymic mice by co-administration of ftorafur and uracil. Int J Radiat Oncol Biol Phys 10:1687-1691, 1984.
46. GROSSIE VB, JR, and LOO TL. Effect of thymidine on the toxicity and antitumor activity of ftorafur. Cancer Treat Rep 65:1087-1091, 1981.
47. KIRKWOOD JM, ENSMINGER W, ROSOWSKY A, ET AL. Comparison of pharmacokinetics in 5-fluorouracil and 5-fluorouracil with concurrent thymidine infusions in a phase I trial. Cancer Res 40:107-113, 1980.
48. YAMAMOTO J, HARUNO A, YOSHIMURA Y, ET AL. Effect of coadministration of uracil on the toxicity of tegafur. J Pharm Sci 73:212-214, 1984.
49. HARRISON SD, JR, DENINE EP, and GILES HD. Evidence of toxicologic activity of ftorafur independent of conversion to 5-fluorouracil. Cancer Treat Rep 63:1389-1391, 1979.

Metabolic Effects of Gallium Nitrate Administered by Prolonged Infusion[1,2,3,4]

Raymond P. Warrell, Jr,* Marian Isaacs, Craig J. Coonley, Nancy W. Alcock, and Richard S. Bockman[5,6]

Gallium nitrate was recently found to be effective treatment for resistant cancer-related hypercalcemia. In vitro and in vivo experiments have suggested that the drug directly inhibits calcium resorption from bone; however, the overall effects of gallium nitrate on calcium balance were unknown. We have completed metabolic balance studies in four patients who received this drug by prolonged infusion. All patients were in positive calcium balance while receiving the drug. Each patient also showed a substantial decrease in urinary calcium excretion. Serum phosphorus decreased in all four patients. There was no change in phosphorus, sodium, chloride, or magnesium balance or in creatinine clearance. We conclude that prolonged infusions of gallium nitrate reduce urinary calcium excretion and that the hypocalcemic effect of this drug is primarily due to inhibition of calcium resorption from bone. Thus, the drug may prove useful in reducing accelerated bone resorption in patients with bone metastases or chronic cancer-related hypercalcemia. [Cancer Treat Rep 69:653–655, 1985]

Gallium nitrate is a new metal-based compound which is currently being evaluated for its antitumor activity. We recently showed that gallium nitrate administered by prolonged infusion was highly effective treatment for cancer-related hypercalcemia and that the hypocalcemic effect was not associated with cytotoxic effects (1). Our studies also suggested that a primary effect of gallium nitrate was to reduce calcium resorption from bone which also reduced urinary calcium excretion (1,2). Previously, Krakoff et al (3) had reported that injections of gallium nitrate produced marked hypercalciuria (3). To resolve this apparent discrepancy, we evaluated the metabolic effects of gallium nitrate in a series of patients who received the drug in a recent phase II study (4).

METHODS

Metabolic Study

Four patients with advanced malignant lymphoma were hospitalized and received a diet of constant defined calcium, sodium, potassium, phosphorus, magnesium, protein, and caloric content. Calcium intake ranged between 900 and 1350 mg/day. Fluid intake was held constant to ensure a urinary volume of approximately 2000 ml/day. After a period of dietary adjustment, an oral nonabsorbable dye was administered, and when the dye was passed, a metabolic balance study was begun. Patients were studied during the next 3 days as a baseline after which they received a continuous iv infusion of gallium nitrate for 7 days at a daily dose of 300 mg/m^2 as previously described (4). Oral fluid intake was controlled for the extra amount of iv fluid used to administer the drug. Oral dye was readministered at the beginning of the observation period, at the beginning of drug treatment, after 3 days of treatment, and at the end of the infusion. Stool collections were divided into three samples according to passage of these markers. Complete 24-hour urine samples were collected separately. Serum concentrations of sodium, potassium, chloride, calcium, magnesium,

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[3] Presented in part at the Annual Meeting of the American Society of Clinical Oncology, May 1983.

[4] Gallium nitrate was supplied by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

[5] Developmental Chemotherapy, Renal/Physiology, and Endocrinology Services, Department of Medicine, Memorial Sloan-Kettering Cancer Center, and Cornell University Medical College, New York, NY.

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**Reprint requests to:* Dr. Raymond P. Warrell, Jr, Department of Medicine, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021.

phosphorus, and creatinine were measured daily. Blood samples for determinations of serum parathyroid hormone (PTH) and 25-hydroxy and 1,25-dihydroxy vitamin D_3 levels were taken at the beginning and end of the drug infusion. Aliquots of each 24-hour urine collection were analyzed for creatinine, sodium, calcium, magnesium, and phosphorus concentration. Stool collections were homogenized and ashed. Aliquots of the ash were dissolved in 0.5 N HNO_3 and were analyzed for calcium, phosphorus, magnesium, sodium, and chloride content. Metabolic balances were calculated according to standard methods (5).

Chemical Analysis

Calcium and magnesium were measured by atomic absorption spectrophotometry; sodium and potassium were measured using flame-emission spectrophotometry; chloride was measured with a Cotlove chloridometer; inorganic phosphorus was measured using a modification of a standard colorimetric method (6); ionized calcium was measured with an ion-specific electrode (Nova Biomedical, Newton, MA); serum creatinine was measured by Fuller's earth method (7); and urine creatinine was measured using the Gemini Creatinine Reagent Kit (Electronucleonics Inc, Fairfield, NJ). 25-Hydroxy and 1,25-dihydroxy vitamin D_3 were separated by high-pressure liquid chromatography and were measured by competitive protein-binding assay (Bio-Science, Great Neck, NY). Carboxy-terminal/mid-region PTH was measured by commercial radioimmunoassay (Smith Kline Clinical Laboratories, Creve Coeur, MO).

RESULTS

Over the 7-day treatment period, the four patients showed minimal changes in ionized serum calcium concentration (table 1). A substantial decrease in serum phosphorus concentration occurred in all patients and was associated with an increase in serum levels of PTH. There was no change in serum levels of 25-hydroxy or 1,25-dihydroxy vitamin D_3.

All four patients were in positive calcium balance during the 7-day drug infusion (table 2). Urinary calcium excretion was lower during the treatment period relative to baseline in all four patients. Fecal calcium excretion was unchanged. There were no major or consistent changes in serum concentrations, urinary excretion, or metabolic balances of sodium, chloride, potassium, or magnesium. Creatinine clearance and phosphorus balance were similarly unchanged from baseline.

DISCUSSION

Although gallium nitrate can cause a profound reduction in serum calcium concentration in hypercalcemic patients (1), the drug produced only minor changes in ionized serum calcium in these patients who were relatively normocalcemic at the initiation of this study. The most significant observation was the consistent and substantial decrease in urinary calcium excretion accompanied by an overall positive calcium balance, which increased progressively during the period of drug treatment. Thus, these patients were continuing to retain more calcium than they excreted.

Krakoff et al (3) had previously reported that gallium nitrate induced marked hypercalciuria. This apparent discrepancy is probably due to two factors, both of which relate to the method of drug administration. First, Krakoff et al administered brief injections accompanied by mannitol and large amounts of iv fluids. This method would have been expected to produce a burst of hypercalciuria due to an osmotic diuresis from the drug and the mannitol. Second, gallium nitrate is currently formulated with 1.15 mg of trisodium citrate/mg of gallium nitrate. Since citrate readily chelates with calcium, rapid injections of this formulation would have produced prompt excretion of calcium citrate. Conversely, the method of prolonged infusion without excessive hydration (4) eliminates the osmotic diuresis and would allow metabolism of citrate by normal biochemical processes.

We conclude that lower daily doses of gallium nitrate administered over extended periods of time reduce urinary calcium excretion and favor a positive calcium balance. Our preliminary data from x-ray diffraction studies suggest that gallium is incorporated into bone mineral

TABLE 1.—Effects of gallium nitrate in patients on metabolic study[*]

Patient No.	Ionized calcium (mmols/L)		Phosphorus (mg/dl)		PTH (pg/ml)		25-Hydroxy vitamin D_3 (ng/ml)		1,25-Dihydroxy vitamin D_3 (pg/ml)	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
1	1.15	1.11	3.9	2.5	187	589	7	6	47	115
2	1.10	1.12	4.2	3.7	121	334	16	16	72	26
3	1.23	1.19	4.4	2.5	413	785	17	16	45	33
4	1.24	1.20	3.9	3.3	117	279	26	19	31	41

[*] Ranges of normal serum concentration are as follows: $N = 1.19-1.33$, ionized calcium; $N = 3.0-4.5$, phosphorus; $N = 60-450$, C-terminal/mid-region PTH; $N = 10-55$, 25-hydroxy vitamin D_3; and $N = 20-76$, 1,25-dihydroxy vitamin D_3.

TABLE 2.—Effects of gallium nitrate infusion on calcium balance and daily fecal and urinary calcium excretion*

Patient No.	Mean daily calcium balance				Mean daily urinary calcium				Mean daily fecal calcium			
		Infusion				Infusion				Infusion		
	Baseline	D0-2	D3-6	Mean 7D	Baseline	D0-2	D3-6	Mean 7D	Baseline	D0-2	D3-6	Mean 7D
1	−447	−166	+162	+22	238	184	94	132	882	625	271	448
2	+297	−55	+164	+70	289	291	210	245	514	854	716	785
3	+43	+331	+489	+421	174	178	102	134	653	361	280	321
4	+230	+151	+160	+156	123	91	43	63	439	550	590	570

* All 4 patients are in positive calcium balance with reduction in total serum calcium concentration and reduced urinary calcium excretion during the drug infusion (data expressed as mg/day of total calcium). D = days of gallium infusion.

and stabilizes or promotes the formation of calcium-hydroxyapatite crystals which are less susceptible to resorption (8). Thus, this agent may ultimately be useful to reduce accelerated bone resorption in cancer patients with bone metastases or chronic cancer-related hypercalcemia (9).

REFERENCES

1. WARRELL RP, JR, BOCKMAN RS, COONLEY CJ, ET AL. Gallium nitrate inhibits calcium resorption from bone and is effective treatment for cancer-related hypercalcemia. J Clin Invest 73:1487-1490, 1984.
2. WARRELL RP, JR, BOCKMAN RS, STASZEWSKI H, ET AL. Control of cancer-related hypercalcemia by gallium nitrate: results of a pilot clinical study. Proc ASCO 2:92, 1983.
3. KRAKOFF IH, NEWMAN RA, and GOLDBERG RS. Clinical toxicologic and pharmacologic studies of gallium nitrate. Cancer 44:1722-1727, 1979.
4. WARRELL RP, JR, COONLEY CJ, STRAUS DJ, ET AL. Treatment of patients with advanced malignant lymphoma using gallium nitrate administered as a seven-day continuous infusion. Cancer 51:1982-1987, 1983.
5. REIFENSTEIN EC, JR, ALBRIGHT F, and WELLS SL. The accumulation, interpretation, and presentation of data pertaining to metabolic balances, notably those of calcium, phosphorus, and nitrogen. J Clin Endocrinol Metab 5:367-395, 1947.
6. SUMNER JB. A method for the colorimetric determination of phosphorus. Science 100:413-414, 1944.
7. OWEN JN, IGGO B, SCANDRETT TJ, ET AL. Plasma or serum creatinine using Fullers earth. Biochem J 58:426-437, 1954.
8. BOCKMAN RS, WARRELL RP, JR, ALCOCK NW, ET AL. Gallium blocks accelerated bone resorption in patients with metastatic cancer and increases calcium content of bone. Clin Res 33:576A, 1985.
9. WARRELL RP, JR, BOCKMAN RS, ALCOCK NW, ET AL. Gallium nitrate reduces accelerated bone turnover in patients with bone metastasis. Proc ASCO 4:42, 1985.

Pharmacokinetics of Hexamethylmelamine Administered via the Ip Route in an Oil Emulsion Vehicle[1,2]

Aeron D. Wickes and Stephen B. Howell[3,4,*]

Saline and Intralipid were compared as vehicles for the ip administration of hexamethylmelamine (HMM) in mice. The drug proved stable over at least 7 weeks in solution and 2 years in crystal form. Fiftyfold higher concentrations of HMM could be achieved in Intralipid than in saline. The peritoneal pharmacokinetics were first-order and linear over the concentration range of HMM in Intralipid from 50 to 2000 μg/ml. The mean peritoneal half-life of HMM in Intralipid was 12.5-fold greater than the mean half-life of HMM in saline at the same concentration. Peritoneal concentration × time drug exposure, as measured by the area under the curve in single-dose elimination experiments, was 1200-fold greater for HMM at 2000 μg/ml in Intralipid than at 50 μg/ml, near saturation, in saline. The steady-state peritoneal to plasma concentration ratios were 96–104 for HMM in Intralipid at concentrations of 300–2000 μg/ml, and the peritoneal concentration that could be maintained by the constant ip infusion of HMM at 2000 μg/ml in Intralipid was at least 1600-fold greater than that maintainable with HMM in saline. The mean peritoneal clearance calculated by two independent methods and at three different concentrations of HMM in Intralipid was 0.112 ± 0.016 ml/minute. HMM in Intralipid is a stable formulation that can be used to increase peritoneal exposure to HMM and may potentially be used iv as well. [Cancer Treat Rep 69:657–662, 1985]

Ovarian carcinomas remain localized to the peritoneal cavity throughout most of their natural history (1,2). Pharmacokinetic modeling (3) has suggested that intracavitary administration of chemotherapeutic agents by peritoneal dialysis may result in a much greater total drug exposure for the intracavitary tumor than for the systemic circulation. The rate at which the chemotherapeutic agent is cleared from the peritoneal cavity, relative to its clearance from blood, is the major determinant of how much greater concentration × time exposure can be delivered to the tumor than to the systemic circulation (3). The usefulness of intracavitary chemotherapy has already been established in the treatment of several tumors confined to extravascular cavities, such as meningeal leukemia (4–8) and early-stage bladder carcinoma (9,10). Initial pharmacologic studies have now been completed for several chemotherapeutic agents administered by the ip route, including 5-FU (11,12), doxorubicin (13), methotrexate (14,16), melphalan (17), and cytarabine (18). In general, these studies have demonstrated (*a*) that all of these agents can be administered by the ip route without serious acute local toxicity and (*b*) that drug concentrations in the peritoneal cavity can be maintained at levels 1–3 logs higher than those in the plasma, thus confirming the predictions made by the pharmacokinetic model (3).

Hexamethylmelamine (HMM) has several properties which make it an attractive candidate for use via the intracavitary route. It is a drug with established activity against ovarian carcinoma (19–21), and it is non-cross-resistant with alkylating agents (22,23). However, HMM is a nonpolar molecule and thus only slightly soluble in aqueous medium, so that there is no parenteral formulation of the drug generally available. In addition, compounds with high lipid-water partition coefficients were found to be rapidly cleared from the peritoneal cavity in

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[3] Department of Medicine and the Cancer Center, University of California-San Diego, La Jolla.

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[*] *Reprint requests to:* Stephen B. Howell, MD, Division of Hematology/Oncology, Department of Medicine T-012, University of California-San Diego, La Jolla, CA 92093.

murine studies (4). We have investigated the use of an oil emulsion vehicle, Intralipid, to increase the concentration of HMM achievable in the peritoneal cavity, and thus to expose tumor cells to higher concentrations of this chemotherapeutic agent. We hypothesized that oil emulsion would also be more slowly absorbed from serosal cavities than aqueous media, and may thus act as a depot which slowly releases locally high concentrations of HMM (5). We present here preclinical studies on the suitability of Intralipid as a vehicle for the ip administration of HMM.

METHODS

HMM was provided as a pure powder by the Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Vehicles of drug administration included 0.9% NaCl in water and Intralipid (Cutter Laboratories, Inc, Berkeley, CA), which is an emulsion containing 10% soybean oil. All animal experiments were performed on BDF_1 (C57BL/6 × DBA/2) mice. Animals were anesthetized for short periods with ether or, when 10-60-minute periods of anesthesia were necessary, with 0.125 ml of 20% nembutal in phosphate-buffered saline. For the high-pressure liquid chromatography (HPLC) assay of HMM, samples were extracted with ten volumes of ethyl acetate evaporated to dryness and reconstituted in 100% methanol (26). Samples were assayed isocratically on a Waters Associates liquid chromatograph at 254 nm using a C-18 μBondapak column and a mobile phase of 0.01 M ammonium formate (pH 3.5):methanol (40:60) at 1.5 ml/minute (22). Concentrations were determined by comparison to an external standard prepared in the same medium (plasma, saline, or Intralipid). The column was washed with 100% methanol for 10 minutes between injections.

To determine solubility of HMM in saline and Intralipid, excess crystalline HMM was mixed for 12 hours in the vehicles; the mixture was centrifuged at 2000 rpm for 5 minutes to remove crystals without causing phase separation of the Intralipid, and the supernatant was filtered through Whatman #1 filter paper and then assayed. The relative solubilities of HMM in the lipid and the aqueous phases of Intralipid were determined by adding tritiated thymidine to HMM in Intralipid. The Intralipid separated into an aqueous layer and a lipid supernatant layer after 5 minutes of centrifugation on an Eppendorf 5414 microcentrifuge (Brinkman Instruments, Westbury, NY). The lipid and aqueous layers were then assayed for HMM concentration and counts per minute per volume. Since thymidine is negligibly soluble in lipid, counts in the lipid layer were compared with counts per volume in the aqueous layer to determine the percentage of volume of the lipid layer attributable to trapped aqueous phase. The amount of HMM in this volume of aqueous layer was then subtracted from the total amount of HMM in lipid layer to yield the true concentration of HMM in the lipid phase.

Stability of the drug in saline and in Intralipid was determined by preparing drug at various concentrations in the vehicles and then dividing each drug-vehicle combination into aliquots and storing half at room temperature and half at 4°C. The samples were then assayed weekly over a 7-week period.

Single-dose peritoneal elimination half-lives were determined by injecting HMM dissolved in 2.5 ml of vehicle into the peritoneum of a mouse and withdrawing 0.1-ml samples at various times afterward for HPLC assay. There was no evidence of phase separation of the Intralipid in the peritoneal cavity. Constant infusions used in determining steady-state peritoneal and plasma clearance rates were maintained using a Harvard infusion apparatus. The 23-gauge butterfly needles used were inserted into the peritoneum of the animal and sutured to the abdomen to hold the needle in place. A 23-gauge Gelco catheter was also inserted into the peritoneum and sutured to the abdomen. A loading dose of drug in 2.0 ml of vehicle was injected into the peritoneum and the constant infusion begun. At various times afterward void volume and 0.1-ml samples were withdrawn from the catheter for HPLC assay. When steady-state conditions were reached in the peritoneum, enough blood to yield 0.1 ml of plasma was withdrawn into heparinized tubes from the retro-orbital sinus for HPLC assay.

RESULTS

HMM was measured by extraction into ethyl acetate and then quantitated by HPLC. The assay was linear over the concentration range of 0.1-1000 μg/ml, and the regression line for the standard curve passed through the origin. Relative to HMM prepared in methanol, the extraction efficiency of HMM from plasma was 92.5%, and the extraction efficiency from Intralipid was 92.4%. The coefficient of variation between samples was < 11% over the HMM concentration range of 2.5-2500 μg/ml.

The stability of HMM in both saline and Intralipid was tested at room temperature and at 4°C. HMM was dissolved in saline at concentrations of 2.5, 22, and 61.5 μg/ml. Over a period of 48 days, six repeated measurements showed no discernible loss of drug at either temperature. A similar experiment was performed with HMM suspended in Intralipid at concentrations of 18, 220, and 2500 μg/ml, and again the change in drug concentration at either temperature over a period of 48 days was smaller than the variance of the assay. HMM was also stable in methanol at a concentration of 1000 μg/ml for 25 days and for at least 30 months when stored in dry powder form at room temperature.

The maximum concentration of HMM achievable in 0.9% saline was 70 ± 3 μg/ml ($n = 3$). In contrast, the HMM concentration attainable in Intralipid was 3493 ±

64 µg/ml ($n = 3$), and where the lipid and aqueous phases were separated by centrifugation, the drug concentration in the lipid phase was found to be 60 times higher than that in the aqueous phase. The HMM concentration measured in the lipid phase was 6004 ± 555 µg/ml ($n = 3$) versus only 102 ± 11 µg/ml ($n = 3$) in the aqueous phase.

To determine the peritoneal elimination half-life, mice were injected with 2.5 ml of HMM at 50, 300, or 2000 µg/ml in Intralipid or the same volume of HMM at 50 µg/ml in saline. Multiple samples of peritoneal fluid were assayed for HMM concentration over a period of 60 minutes. Four repetitions of each combination were performed. The data presented in figure 1A and B demonstrate first-order elimination kinetics for both vehicles and for all three concentrations of HMM in Intralipid. The half-life of HMM at 50 µg/ml in saline was 2.0 ± 0.7 minutes, as calculated from the mean of four repetitions, using the slope of the best-fit linear regression line for each experiment. The half-life of HMM at the same initial concentration in Intralipid was 25 ± 7 minutes, and the half-lives at 300 and 2000 µg/ml of HMM in Intralipid were 34 ± 5 and 27 ± 6 minutes, respectively. Analysis of variance indicated no significant difference in the peritoneal clearance at the three concentrations of HMM in Intralipid, indicating linear pharmacokinetics over the wide concentration range tested. The difference in the half-life of HMM in saline and in Intralipid at 50 µg/ml was statistically significant ($P < 0.001$, Student's t test).

The area under the peritoneal concentration × time curve (AUC), a measure of total drug exposure for the peritoneal cavity, was calculated from the mean best-fit linear regression line for each single-dose elimination experiment. The AUC was determined by the trapezoid rule from 0 to 30 minutes, then by first-order extrapolation to infinite time. The peritoneal AUCs were 22.5 µg · minute/ml for HMM administered at 50 µg/ml in saline and 610, 5140, and 27,300 µg · minute/ml for HMM in Intralipid at 50, 300, and 2000 µg/ml, respectively.

Constant ip infusions of HMM in each vehicle were used to determine the steady-state peritoneal and plasma concentration and clearance ratios. After a loading dose of HMM in 2 ml of vehicle was administered into the peritoneal cavity, mice were infused ip with HMM in either saline or Intralipid at 0.0136 ml/minute, a rate which maintained a constant peritoneal volume, and multiple peritoneal fluid and plasma samples were obtained to document attainment of steady-state. Figure 2 shows that for HMM concentrations of 50, 300, and 2000 µg/ml in Intralipid, steady-state was reached in the peritoneal cavity by 165 minutes. In three separate experiments involving infusion of HMM at 300 µg/ml in Intralipid, it was documented that steady-state had also been achieved in the plasma; the mean ± SD difference in plasma concentration of HMM at 165 and at 180 minutes was 0.02 ± 0.01 µg/ml. Figure 3 shows that there was a linear relationship between rate of ip infusion of HMM in Intralipid and the steady-state concentration attained in the abdominal cavity. This further confirms the linearity of the

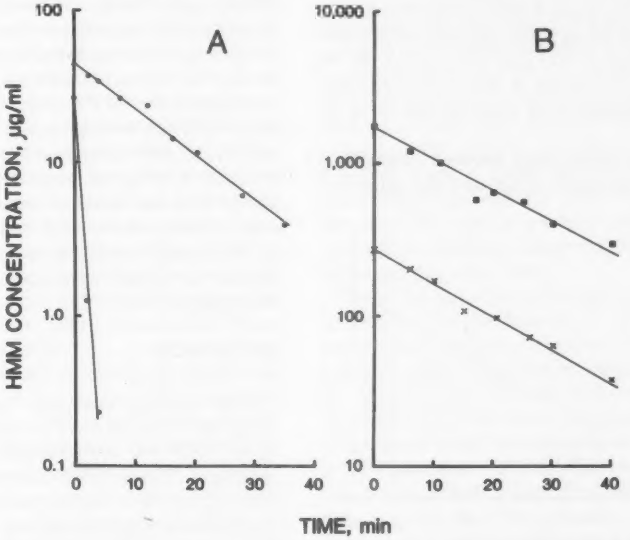

FIGURE 1.—HMM concentration in peritoneal cavity as function of time after injection of 2.5 ml of drug-containing vehicle. A: HMM at 50 µg/ml in 0.9% sodium chloride (●); HMM at 50 µg/ml in Intralipid (○). B: HMM at 300 µg/ml in Intralipid (x); HMM at 2000 µg/ml in Intralipid (■). Data are from a single representative experiment.

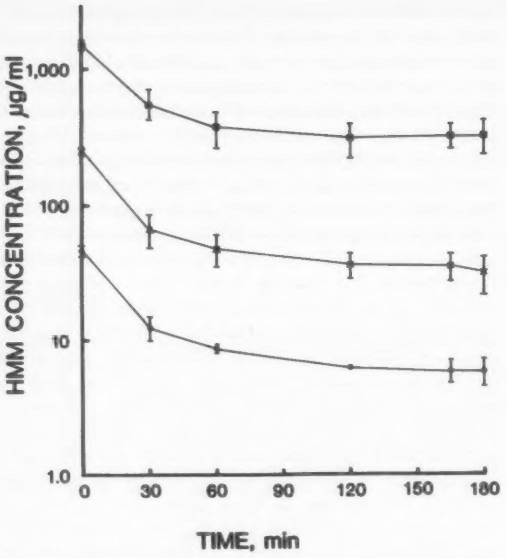

FIGURE 2.—HMM concentration in peritoneal cavity of mice following instillation of 2.0 ml of drug-containing vehicle followed by initiation of constant ip infusion of 0.0136 ml/min. HMM at 50 µg/ml (O); HMM at 300 µg/ml (x); HMM at 2000 µg/ml (■).

ip pharmacokinetics over the wide concentration range studied.

The data presented in table 1 indicate that the mean steady-state peritoneal:plasma HMM concentration ratio ranged from 96 to 104 when HMM was infused in Intralipid at concentrations of 300 and 2000 µg/ml. At an HMM concentration of 50 µg/ml in either saline or Intralipid, the plasma concentrations were too low to be accurately quantified.

At steady-state, the relationship between clearance of the drug from the peritoneal cavity and the peritoneal and plasma concentrations of HMM is given by Equation 1 (17).

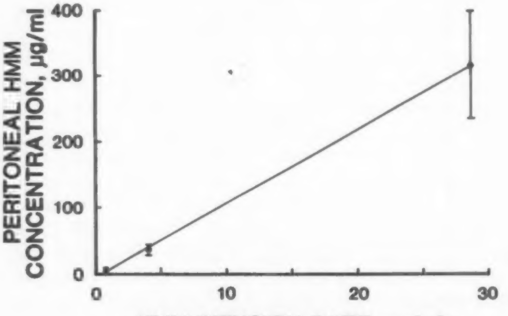

FIGURE 3.—Peritoneal HMM concentration at steady-state during constant ip infusion of HMM in Intralipid. Each data point = mean of 4 experiments; vertical bars = SD.

$$PA = \frac{I}{(HMM)_{ip} - R(HMM)_{pl}} \qquad [1]$$

PA is the peritoneal permeability-area coefficient (equivalent to clearance) in ml/minute; I is the drug infusion rate in µg/minute; $(HMM)_{ip}$ and $(HMM)_{pl}$ are drug concentrations in the peritoneum and plasma, respectively; and R is the plasma protein-binding coefficient of the drug, assumed in this case to be 1. The PA values calculated were 0.12 ± 0.03 ($n = 4$) and 0.10 ± 0.03 ml/minute ($n = 4$) for HMM infused in Intralipid at 300 and 2000 µg/ml, respectively.

At steady-state, systemic clearance (Cl) can be determined from the ratio of peritoneal to plasma HMM concentrations by substituting the PA values calculated from Equation 1 into Equation 2 (3).

$$\frac{(HMM)_{ip}}{(HMM)_{pl}} = \frac{Cl + PA}{PA} \qquad [2]$$

The calculated plasma clearances were 17 ± 11 and 11 ± 7 ml/minute for HMM infused ip at 300 and 2000 µg/ml in Intralipid, respectively.

The peritoneal PA can also be independently determined from the slope of the single-dose elimination curves using Equation 3 (12).

$$\ln (HMM)_t = \ln (HMM)_0 - \frac{PA}{V} t \qquad [3]$$

$(HMM)_t$ and $(HMM)_0$ are the ip drug concentrations at times t and 0, respectively; t is time in minutes and V is the ip volume of drug in milliliters. Using a V of 2 ml (the average ip volume considering loss with sampling), the calculated values of PA were 1.7 ± 0.5 ml/minute for 50 µg/ml of HMM in saline and 0.13 ± 0.04, 0.09 ± 0.01, and 0.12 ± 0.03 ml/minute for HMM in Intralipid at 50, 300, and 2000 µg/ml, respectively. These results agree closely with the values calculated using Equation 1. The mean of the peritoneal PA values calculated from these two independent methods and at three different concentrations of HMM in Intralipid was 0.112 ± 0.016 ml/minute ($n = 5$).

DISCUSSION

HMM is only sparingly soluble in saline, but as expected on the basis of its lipid-water partition coefficient, HMM was approximately 50-fold more soluble in Intralipid. When HMM was suspended in Intralipid at 1000 µg/ml, the resulting concentration of HMM in the lipid phase was 60-fold greater than that in the aqueous phase. At near maximum concentrations in saline, HMM was cleared rapidly from the peritoneal cavity of mice, with a half-life of 2 minutes and a clearance of 1.7 ml/minute. These findings confirm those of Torres et al

TABLE 1.—Steady-state peritoneal and plasma HMM concentrations during constant infusion*

Vehicle	Concentration of HMM infused (μg/ml)	Infusion rate (μg/min)†	Steady-state HMM concentration (μg/ml ± SD)‡		
			Peritoneal	Plasma	Concentration ratio peritoneal/plasma
Intralipid	2000	27.2	318 ± 81	3.3 ± 2.1	96.4
Intralipid	300	4.1	35.4 ± 8.4	0.34 ± 0.20	104.1
Intralipid	50	0.68	5.9 ± 1.2	< 0.2§	> 29.5
Saline	50	0.68	< 0.2§	< 0.2§	—

*Mice injected with ip loading dose of HMM in 20 ml of vehicle prior to constant infusion.

†Vehicles infused at 0.0136 ml/min.

‡Sampled at 165 mins after constant infusion began, $n = 4$ for each vehicle.

§HMM concentrations below the limit of sensitivity of the assay.

(24), who found that drugs with high lipid-water coefficients were rapidly cleared. The clearance of HMM from the peritoneal cavity was reduced dramatically to 0.1 ml/minute, with a half-life of 25 ± 7 minutes when administered in Intralipid at the same concentration (50 μg/ml).

The AUC values calculated from the single-dose elimination experiments demonstrate that the peritoneal cavity can be exposed to much higher concentration × time drug levels when HMM is administered in Intralipid than when administered in saline. The AUC from HMM at 2000 μg/ml in Intralipid was > 1200 times that for HMM at 50 μg/ml; the latter concentration is near the maximum achievable in saline.

The superiority of Intralipid over saline as a vehicle for the ip administration of HMM was also evident from examination of the steady-state peritoneal and plasma concentrations and clearances. The clearance of HMM from plasma was 100-fold more rapid than its peritoneal clearance when the drug was administered in Intralipid. This resulted in 100-fold higher HMM concentrations in the peritoneal cavity than in the plasma at steady-state over a wide range of HMM concentration (300–2000 μg/ml). Although the steady-state peritoneal to plasma concentration and clearance ratio for HMM in saline at 50 μg/ml could not be accurately determined, it was clear that only very low peritoneal levels could be maintained when HMM was administered in saline. When HMM in Intralipid at 2000 μg/ml was instilled ip, the peritoneal steady-state concentration was at least 1600-fold higher than the concentration that could be maintained when HMM was administered in saline.

The decay of HMM concentrations in the peritoneal cavity was first-order for both vehicles. With Intralipid the ip kinetics were linear over a wide concentration range up to 2000 μg/ml. This linearity was noted in both single-dose elimination and constant-infusion experiments, and indicates that even at the higher concentrations tested the clearance mechanism was not saturated.

The exact mechanism by which HMM causes lethal damage is not known (28). It has been demonstrated that HMM can be metabolized intracellularly to pentamethylmelamine (29), and that the activity of both HMM and pentamethylmelamine is enhanced by the action of liver microsomes (28). However, if activation is absolutely required, this may well involve conversion in the target tumor cell rather than involving activation of the liver followed by transfer of a reactive intermediate through the plasma to the tumor cell. It remains to be determined whether HMM injected directly into the peritoneal cavity can cause significant cytotoxicity to human ovarian carcinoma cells. In any case, the results presented in this paper indicate that it is possible to formulate HMM in a stable form in Intralipid in amounts that are satisfactory for use either iv or ip.

It is not clear from this study what accounts for the reduction of peritoneal clearance when HMM is suspended in Intralipid as opposed to saline. One likely explanation is that HMM in the aqueous phase of the Intralipid is cleared as rapidly as is HMM in saline, but that a relatively slow release of HMM from the lipid phase of the Intralipid limits the total flux of drug from the peritoneal cavity. Another uncertainty is whether tumor cells in the peritoneal cavity are exposed only to the HMM concentration that is present in the aqueous phase of the Intralipid, or whether there is direct lipid phase to cell membrane transfer of drug at much higher concentrations. However, even if tumor cells are exposed to only the lower HMM concentration present in the aqueous phase, the use of Intralipid as a vehicle offers the opportunity to maintain the HMM concentration in the peritoneal cavity for longer periods of time.

One of the major limitations of ip chemotherapy for the treatment of such tumors as ovarian carcinoma and mesothelioma is likely to be the relatively poor tissue diffusion of most water-soluble antitumor drugs. Lipid-soluble drugs can be expected to demonstrate better tissue penetration, but the rapid peritoneal clearance of these drugs limits the differential peritoneal versus plasma exposure that can be achieved. The results of this study suggest that if HMM is directly activated by target tumor cells, then the use of Intralipid as a vehicle may surmount this problem and permit better tissue penetration than can be achieved with water-soluble drugs. It remains to be determined whether Intralipid could serve the same function for other lipid-soluble drugs such as some of the

nitrosoureas, which can also be formulated in lipid emulsions (30) and clearly work directly on tumor cells without a requirement for activation in another tissue.

REFERENCES

1. TOBIAS JS, and GRIFFITHS CF. Management of ovarian carcinoma. Current concepts and future prospects. N Engl J Med 294:818-822, 1976.
2. KATZ ME, SCHWARTZ PE, KAPP DS, ET AL. Epithelial carcinoma of the ovary: current strategies. Ann Intern Med 95:98-111, 1981.
3. DEDRICK RL, MYERS CE, BUNGAY PM, ET AL. Pharmacokinetic rationale for peritoneal drug administration in the treatment of ovarian cancer. Cancer Treat Rep 62:1-11, 1978.
4. HYMAN CB, BOGLE JM, BRUBAKER CA, ET AL. Central nervous system involvement by leukemia in children. II. Therapy with intrathecal methotrexate. Blood 25:13-22, 1965.
5. BAND PR, HOLLAND JF, BERNARD J, ET AL. Treatment of central nervous system leukemia with intrathecal cytosine arabinoside. Cancer 32:744-748, 1973.
6. BLEYER WA, DRAKE JC, and CHABNER BA. Pharmacokinetics and neurotoxicity of intrathecal methotrexate therapy. N Engl J Med 289:770-774, 1973.
7. BLEYER WA, and DEDRICK RL. Clinical pharmacology of intrathecal methotrexate. I. Pharmacokinetics in nontoxic patients after lumbar injection. Cancer Treat Rep 61:703-708, 1977.
8. BLEYER WA, POPLACK DG, and SIMON RM. "Concentration × time" methotrexate via a subcutaneous reservoir: a less toxic regimen for intraventricular chemotherapy of central nervous system neoplasms. Blood 51:835-842, 1978.
9. VEENEMA RJ, DEAN AL, JR, ROBERTS M, ET AL. Bladder carcinoma treated by direct instillation of thio-TEPA. J Urol 88:60-63, 1962.
10. NIEH PT, DALY JJ, HEANEY JA, ET AL. The effect of intravesical thio-tepa on normal and tumor urothelium. J Urol 119:59-61, 1978.
11. SPEYER JL, and MYERS CE. The use of peritoneal dialysis for delivery of chemotherapy to intraperitoneal malignancies. Recent Results Cancer Res 74:264-269, 1980.
12. SPEYER JL, COLLINS JM, DEDRICK RL, ET AL. Phase I pharmacological studies of 5-fluorouracil administered intraperitoneally. Cancer Res 40:567-572, 1980.
13. OZOLS RF, YOUNG RC, SPEYER JL, ET AL. Phase I and pharmacologic studies of adriamycin administered intraperitoneally to patients with ovarian cancer. Cancer Res 42:4265-4269, 1982.
14. HOWELL SB, CHU BCF, WUNG WE, ET AL. Long-duration intracavitary infusion of methotrexate with systemic leucovorin protection in patients with malignant effusions. J Clin Invest 67:1161-1170, 1981.
15. JONES RB, MYERS CE, GUARINO AM, ET AL. High volume intraperitoneal chemotherapy ("belly bath") for ovarian cancer. Cancer Chemother Pharmacol 1:161-166, 1978.
16. JONES RB, COLLINS JM, MYERS CE, ET AL. High-volume intraperitoneal chemotherapy with methotrexate in patients with cancer. Cancer Res 41:55-59, 1981.
17. HOWELL SB, PFEIFLE CE, and OLSHEN RA. Intraperitoneal chemotherapy with melphalan. Ann Intern Med 101:14-18, 1984.
18. KING ME, and HOWELL SB. Intraperitoneal cytarabine therapy in ovarian carcinoma. J Clin Oncol 2:662-669, 1984.
19. WILSON WL, SCHROEDER JM, HUMMEL RP, ET AL. Phase II study of hexamethylmelamine. Cancer 23:132-136, 1969.
20. LEGHA SS, SLAVIK M, and CARTER SK. Hexamethylmelamine, an evaluation of its role in the therapy of cancer. Cancer 38:27-35, 1976.
21. WAMPLER GL, MELLETTE SJ, KUPERMINC M, ET AL. Hexamethylmelamine (NSC-13875) in the treatment of advanced cancer. Cancer Chemother Rep 56:505-514, 1972.
22. BONOMI PD, MLADINEO J, MORRIN B, ET AL. Phase II trial of hexamethylmelamine in ovarian carcinoma resistant to alkylating agents. Cancer Treat Rep 63:137-138, 1979.
23. JOHNSON BL, FISHER, RI, BENDER RA, ET AL. Hexamethylmelamine in alkylating agent-resistant ovarian carcinoma. Cancer 42:2157-2161, 1978.
24. TORRES IJ, LITTERST CL, and GUARINO AM. Transport of model compounds across the peritoneal membrane in the rat. Pharmacology 17:330-340, 1978.
25. TAKAHASHI T, UEDA S, MAJIMA S, ET AL. Attempt at local administration of anticancer agents in the form of fat emulsion. Cancer 38:1507-1514, 1976.
26. MORIMOTO M, GREEN D, SCHEIN PS, ET AL. Comparative pharmacology of pentamethylmelamine and hexamethylmelamine in mice. Cancer Res 40:2762-2767, 1980.
27. BENVENUTO JA, STEWART DJ, LOO TJ, ET AL. Pharmacology of pentamethylmelamine in humans. Cancer Res 41:566-569, 1981.
28. RUTTY CJ, and CONNORS TA. In vitro studies with hexamethylmelamine. Biochem Pharmacol 26:2385-2391, 1977.
29. BEGLEITER A, GOVER J, and GOLDENBERG GJ. Uptake and metabolism of hexamethylmelamine and pentamethylmelamine by L5178Y lymphoblasts in vitro. Cancer Res 40:4489-4494, 1980.
30. DAVIGNON JP, YANG KW, WOOD HB, JR, ET AL. Formulation of three nitrosoureas for intravenous use. Cancer Chemother Rep Part 3, vol 4(3):7-11, 1973.

Evidence for a Novel Hybrid Immunotoxin Recognizing Ricin A-Chain by One Antigen-Combining Site and a Prostate-Restricted Antigen by the Remaining Antigen-Combining Site: Potential for Immunotherapy[1,2]

Karen S. Webb,[3,*] Joy L. Ware,[3] Susanne F. Parks,[3,4] Philip J. Walther,[3,4] and David F. Paulson[3,4]

We have used cell fusion technology to produce second-generation hybridomas which secrete a population of murine monoclonal antibodies (MABs), a portion of which are bifunctional antibodies. The bifunctional hybrid MABs produced are capable of recognizing ricin A-chain (RAC) via one antigen-combining site and a prostate-restricted antigen via the other antigen-combining site of the IgG molecule. The second-generation hybridoma described in this report resulted from the fusion of spleen cells from mice immunized with purified RAC to hybridoma cells which secrete prostate-directed α Pro 15 monoclonal antibody. We have demonstrated that the MAB population secreted by the second-generation hybridoma can be physicochemically separated into distinct populations exhibiting differential binding to the cultured prostatic carcinoma cell surface and to RAC immobilized in a solid phase; specifically, a subset of the monoclonal antibody population which exhibits high binding to both prostatic carcinoma cells and to solid-phase RAC can be enriched by physicochemical methods. Binding of hybrid immunotoxin (HIT) MAB population to RAC can be quantitatively reduced by prior adsorption of the antibody population with prostate carcinoma cells; conversely, hybrid MAB binding to prostate carcinoma cells can be quantitatively reduced by prior adsorption with RAC. The biologic impact of the HIT has been evaluated by the ability of the HIT-RAC conjugate to reduce the uptake of ^{14}C-amino acids into cellular protein. This effect is selective, since HIT-RAC conjugates do not exert an effect on labeled amino acid uptake by a cell line that does not express the target antigen recognized by the prostate-directed component of the hybrid MAB. Furthermore, depression of labeled amino acid uptake by prostate carcinoma cells exhibits a stoichiometric relationship with respect to both the concentration of HIT-MAB and to RAC to which the cells are exposed. The biologic impact of HIT-RAC conjugates on prostate carcinoma cells is enhanced markedly in the presence of lysosomotropic amines. [Cancer Treat Rep 69:663–672, 1985]

The concept of tumor site-directed delivery of toxic agents was initiated early in the century by Ehrlich (5), later revived in the 1950s by the experiments of Mathé et al (10), and has undergone a recent intensive resurgence. In the past decade, numerous investigators have employed murine monoclonal antibodies (MABs) directed to various tumor-restricted antigens as vehicles for site-directed transport of highly toxic agents (1,2,7,16,20). The dimeric structure of ricin, a toxin derived from the castor bean, is highly amenable to immunotoxin construction, since the toxic A-chain moiety can be separated from the binding B-chain subunit. The tumor-directed MAB is substituted for the binding subunit of ricin, thus conferring tumor specificity to the toxic A-chain.

With the exception of work carried out by Raso and Griffin (17), all investigations to date have employed chemical coupling methods, using reagents that promote covalent coupling of ricin A-chain (RAC) to tumor-

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[3] Department of Surgery, Division of Urology, Duke University Medical Center, Durham, NC.

[4] Veterans Administration Hospital, Durham, NC.

[*] *Reprint requests to:* Dr. Karen S. Webb, Box 3062, Department of Surgery, Division of Urology, Duke University Medical Center, Durham, NC 27710.

directed MAB or using bifunctional cross-linking reagents to couple toxin to MAB (8). However, such chemical manipulations frequently disturb the biologic integrity of the MAB component by alteration of the antigen-binding site and/or alteration of the effector function of the toxic A-chain component. It is becoming increasingly apparent that tumor site-directed transport of toxins is a necessary, but not sufficient, prerequisite for killing of tumor cells. Since the binding moiety of the toxin has been replaced by MAB, the toxic A-chain must be transported across the cell membrane either alone or complexed with MAB; the linkage between toxic A-subunit and immunotoxin must be cleaved and the A-chain must be transported through the intracellular membrane compartment to reach the cytosol and inactivate ribosomal protein synthesis (8,20).

The hybrid immunotoxin (HIT) described in this report is not derived by chemical linkage; the linkage of RAC to prostate-directed MAB is by conventional antigen-antibody interactions (fig 1) and, thus, poses no interference with the biologic function of either antibody or toxin.

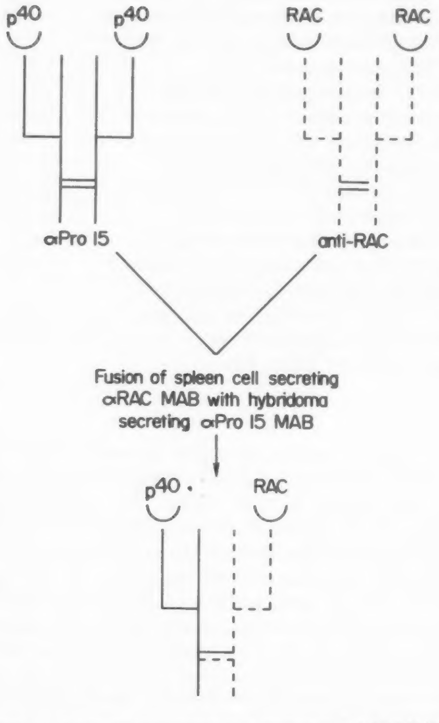

FIGURE 1.—Schematic representation of generation of hybrid MAB. Immune spleen cells secreting antibody to RAC were fused with a hybridoma secreting prostate-directed MAB *α* Pro 15. Assuming random assortment of heavy and light chains, the proportion of MAB with dual specificity should constitute ⅛ of the total combinations possible.

The precedent for producing second-generation (double) hybridomas was established in 1973 by Cotton and Milstein (4). Raso and Griffin (17) demonstrated that HITs could serve as a vehicle for RAC-mediated cytotoxicity. The HIT investigated by Raso and Griffin, however, was not the product of a second-generation hybridoma, but was produced by reduction of rabbit polyclonal IgG antibodies raised versus RAC and human IgG; heavy and light chains of the monomeric fragments were reconstituted to produce hybrid antibody. Binding of HIT at the tumor cell surface is univalent. Antibodies which have been rendered univalent have been reported to achieve a higher surface density than their divalent counterparts (3), a property which would be highly desirable in specific immunotherapy using immunotoxins since the literature reveals that a threshold level of cell surface target antigen expression must exist for a cell to be killed by a given immunotoxin (2,12) and, with rare exception (23), antigen density must be high. Based on optimal expression of any given cell surface, tumor-associated molecule, Neville and Youle (13) used kinetic data to show that a single immunotoxin will not maximally affect cell kill. It is possible that immunotoxins restricted to univalent binding may be able to load at the surface at a density that would allow optimal cell kill. However, multiple factors, other than univalent binding of immunotoxin, must also be considered. Whether or not the antigen-antibody complex is endocytosed under conditions of univalent binding is an important parameter and the extent of endocytosis may vary among different target antigen-immunotoxin systems. Under conditions where divalent prostate tumor-directed antibody was most likely restricted to univalent binding, maximal endocytosis was observed (22). However, Youle and Neville (26) observed that immunotoxins rendered univalent by constructing F(ab)′ fragments were less toxic than immunotoxins constructed with intact monoclonal anti Thy 1.1 antibody.

The HIT reported here also differs from conventional chemically coupled immunotoxins in that the RAC molecule is closely juxtaposed to the cell surface by virtue of its position on the antigen-binding site of the hybrid MAB. Last, the effectiveness of HIT IgG in depressing cellular protein synthesis in the experiments reported here must be evaluated in the context that pure HIT MAB was not used in the experiments. Purified immunoglobulin was used but the IgG population consisted of parental *α* Pro 15 and anti-RAC MABs in addition to nonfunctional and functional hybrid recombinants. Assuming absolute random association of *α* Pro 15 and anti-RAC MAB heavy and light chains within the intracellular pool, one-eighth of the total IgG population should consist of functional hybrid recombinants. With this stipulation, the biologic impact of total IgG compares favorably with that of conventional immunotoxins. The HIT described in this report has potential both as an alternative model to evaluate tumor cell kill by immunotoxins generated by chemical coupling methodology and as a tool for the clinical arena.

PRODUCTION OF SECOND-GENERATION HYBRIDOMAS

BALB/c mice were immunized ip with 20 μg of purified RAC (E-Y Laboratories) every 2 weeks for three immunizations. The first immunization used Freund's complete adjuvant and the remaining two ip immunizations were carried out using incomplete adjuvant. Mice received 10 μg of RAC in phosphate-buffered saline by caudal vein injection 1 week after the third ip immunization and 3 days prior to fusion. The immunization protocol described was known to be effective in generating a high yield of spleen cells producing high-titer MAB to RAC (24). Spleen cells were fused either to hybridoma cells secreting prostate tissue-directed *α* Pro 13 monoclonal antibody (23) or to cells secreting *α* Pro 15 monoclonal antibody using a spleen:hybridoma ratio of 2:1, essentially according to the method of Oi and Hertzenberg (14). *α* Pro 15 monoclonal antibody exhibits a target cell reactivity and specificity identical to that of prostate-directed MAB *α* Pro 13; preliminary experiments indicate that *α* Pro 15 recognizes a different epitope on the same p40 prostate-restricted molecule as that recognized by *α* Pro 13 (K. S. Webb, unpublished observations).

Following fusion, cells were seeded at a density of 4 × 10^4 cells/well. Nonimmune mouse spleen cells were used as a feeder layer. Approximately 1000 wells from master plates representing the products of four separate fusions involving either *α* Pro 13 or *α* Pro 15 hybridomas were screened for ability to bind to both solid-phase immobilized RAC and to PC-3 cells (9). All wells of the master plate exhibited growth that was evident after 1 week in culture. Of these 1000 wells, 75 contained supernatant that exhibited dual binding to PC-3 and RAC. Of the original 75 wells containing cells producing antibody capable of dual PC-3 and RAC binding after 1 week in culture, dual reactivity persisted and could not be cloned out in 18 wells. Thus, approximately 2% of the wells seeded contained double hybridomas that were able to be maintained in culture.

EVIDENCE FOR PRODUCTION OF HIT-MAB

Screening of Hybrids for Dual Reactivity to PC-3 Cells and to RAC

Supernatants from fusion wells containing viable hybrids were tested for ability to bind to cultured prostatic carcinoma PC-3 (9) cells in a cellular-binding assay as previously described (21) and for ability to bind to RAC immobilized in a solid-phase assay (23). Cells from wells containing supernatant binding to both PC-3 cells and to RAC were cloned by limiting dilution and clones were again tested for ability to bind to PC-3 cells and to solid-phase RAC. It should be noted that *continued* synthesis of MAB reactive with RAC is attributable to immortalization of anti-RAC antibody production by fusion of spleen cells with the Sp2-derived *α* Pro 15 hybridoma; spleen cells are not able to survive in culture during the time encompassed by two clonings. Those clones exhibiting dual reactivity were further investigated. All second-generation hybridomas reported here have been cloned at least twice. One criterion for the existence of second-generation hybridomas secreting HITs resides in the inability to "clone out" dual reactivity to PC-3 cells and to RAC. The results of a typical screening assay of the MAB produced by an anti-RAC *α* Pro 15 second-generation hybridoma are shown in figure 2A. It is apparent that

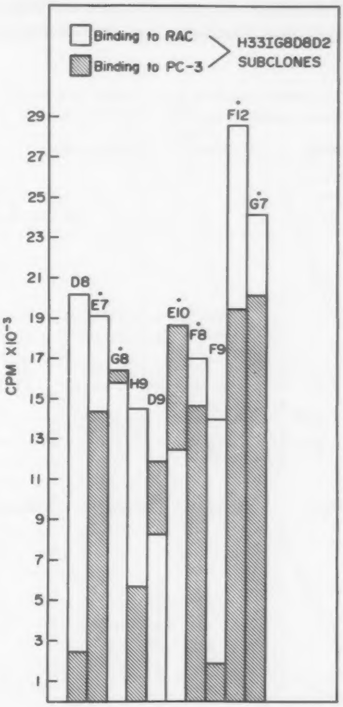

FIGURE 2A.—Differential binding of supernatants from subclones of HIT hybridoma H33IG8D8D2 to cultured prostatic PC-3 carcinoma cells in a cellular-binding assay vs RAC in a solid-phase assay. RAC was immobilized to a 96-well flexible microtiter plate for 12 hrs at 37°C at a concentration of 10 μg of RAC/well. Unsaturated binding sites were subsequently blocked by incubation for 30 mins with a phosphate-buffered saline (PBS)/2% BSA rinse buffer. Immobilized RAC was then fixed with absolute MeOH for 5 mins and subsequently washed 2 times with the rinse buffer prior to addition of clonal supernatants for assay. Binding to PC-3 cells and to solid-phase RAC was quantitated by an indirect method using antimouse Ig and iodinated protein A (18,20). Binding controls consisted of: (*a*) exposure of parental *α* Pro 15 hybridoma supernatant to PC-3 cells (positive control) and to immobilized solid-phase RAC (negative control), and (*b*) exposure of anti-RAC MAB to PC-3 cells (negative control) and to solid-phase RAC (positive control).

differential reactivity to PC-3 cells and to the RAC molecule is obtained on testing of each clonal supernatant. Reactivity varies slightly with regard to passage, but is most pronounced from clone to clone. This is exemplified by the relatively fast-kill kinetics of clone H33IG8D8D2 (fig 3), as opposed to the slower kinetics of the clones in table 1. This type of variability is expected, since the second-generation HIT hybridoma produces both α Pro 15 and anti-RAC parental antibodies and nonfunctional hybrid antibody. Although random fluctuations in differential MAB reactivity to RAC and to PC-3 for any given clone will be caused by variations in the relative intracellular concentration of anti-RAC and α Pro 15 heavy and light chains that can randomly associate to produce functional HIT-MAB, it is apparent from the data in figure 2A that high dual reactivity can be "cloned in." Those clonal supernatants exhibiting high and approximately equal binding to both PC-3 and RAC are indicated by asterisks. Figure 2B shows typical binding curves generated on testing four clonal derivatives of α Pro 15-derived double hybridoma. Although the assay used to detect bound antibody was indirect, it is nevertheless apparent that HIT-MAB binds to both PC-3 and DU-145 prostate carcinoma cells at a higher level than does parental α Pro 15 MAB. J47, a polyclonal rabbit antiserum raised to RAC and tested versus RAC and versus PC-3 and DU-145 cells, is included as a positive and negative binding control, respectively. The reactivity of

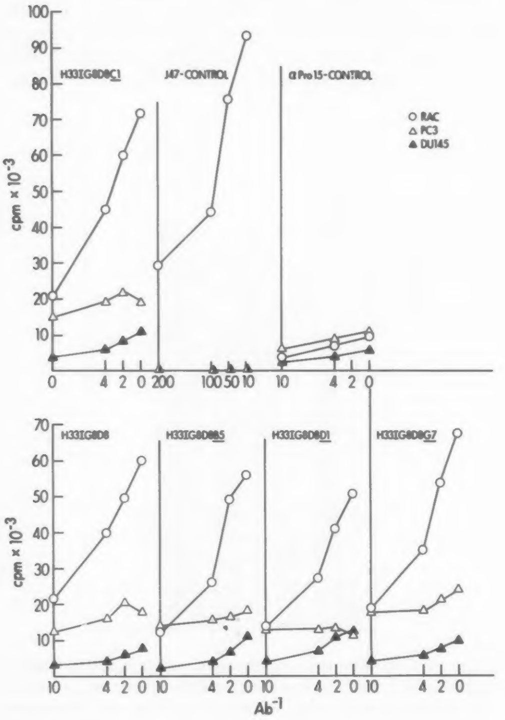

FIGURE 2B.—Binding parameters of hybrid MAB produced by 4 different clonal derivatives of parental clone H33IG8D8. Concentration of IgG for each hybrid MAB preparation was adjusted to 2 mg/ml prior to assay and used at the concentrations indicated. Reaction with a polyclonal antiserum (J47) raised in rabbits against RAC constituted positive binding control; reaction of J47 with PC-3 and DU-145 cells represented negative binding controls. Binding of α Pro 15 to PC-3 and DU-145 cells constituted positive binding controls. Reaction of α Pro 15 with RAC was negative binding control. SE for any given point was generated by triplicate determinations and did not exceed 10% for any given experimental point.

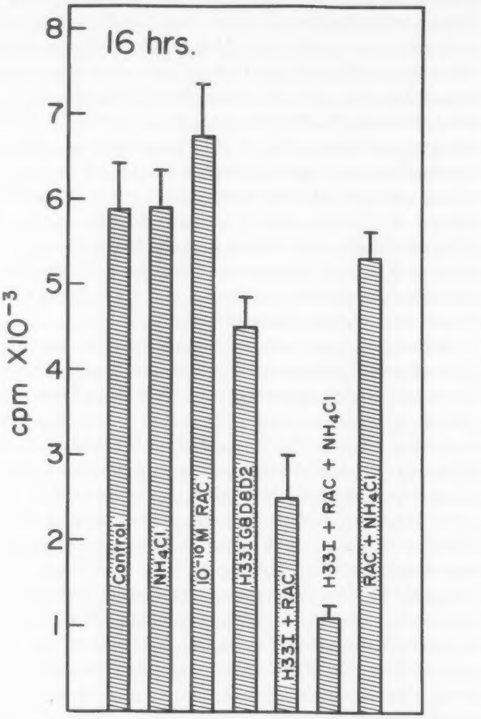

FIGURE 3.—Depression of ^{14}C-amino acid (Amersham U-^{14}C protein hydrolysate; 56 mCi mAtom; CBF 25) uptake in PC-3 prostate carcinoma cells exposed to 50 μg of H33IG8D8D2 antibody alone, in conjunction with 10^{-10} M RAC and with RAC + 10 mM NH_4Cl. Cells at 80% confluence in 96-well microtiter plates (Falcon) were exposed to HIT mixture or to HIT in combination with other reagents. Each experimental parameter was run in quadruplicate for each time point. After 16 hrs of exposure, experimental reagents were removed by aspiration, cells washed once with PBS, and exposed to ^{14}C-amino acids for 8 hrs. After the 8-hr pulse, labeled amino acids were removed, and cells were washed once with PBS, removed from the microtiter well with 0.05% trypsin (0.2 ml), and immediately placed on 0.2 ml ice-cold 20% trichloroacetic acid (TCA). Radiolabeled protein was collected by precipitation on a 96-well microtiter plate containing nitrocellulose filters (Millititer HA plates, Millipore Corp, Bedford, MA). Each filter was washed 3 times with ice-cold 10% TCA. Individual filters were punched out and counted for radioactivity.

parental α Pro 15 to PC-3 and DU-145 cells and to solid-phase immobilized RAC has also been included in figure 2B. It is also apparent that the HIT is more reactive with PC-3 than with DU-145; the parental α Pro 15 antibody exhibits a higher binding to PC-3 cells as compared to DU-145 cells (23).

Separation of Differential Reactivity by Chromatofocusing of MAB Population

The data in figure 4 indicate that the HIT hybridoma synthesizes parental MAB, nonfunctional recombinants, and functional HIT. The basis of differential binding to antigen, summarized in the upper right quadrant of figure 4, is the separation of parental antibody, functional hybrid antibody, and nonfunctional hybrid antibody by the chromatofocusing procedure. Since fraction III (pH 6–7) exhibits high and equal binding to both RAC and to PC-3, chromatofocusing using a narrower pH gradient is currently being used to purify functional HIT from the remainder of the MAB population.

Effect of Cross-Adsorption on Binding Properties of MAB Population

If HIT is being dealt with, one would expect that reactivity of the MAB population to PC-3 cells could be decreased by prior adsorption with RAC; conversely, reactivity of antibody with RAC should be decreased by prior adsorption with PC-3 cells. The data shown in figure 5 substantiate that binding of MAB can be quantitatively reduced by cross-adsorption experiments.[5] It is of note that the observed binding decrease (fig 5) is not attributable to nonspecific trapping. The binding curve of a MAB directed to RAC was qualitatively identical on direct testing and after previous adsorption with PC-3 cells. Because binding of HIT-MAB is being observed in the context of a mixed antibody population, cross-adsorption experiments of the type shown in figure 5 would not be expected to completely abrogate reactivity to either target.

HIT can compete with parental immunotoxin for binding to target antigen on PC-3 cells. This was established by preincubation of subconfluent PC-3 cells with constant amounts of hybrid antibody (100 µg/well) and then challenging with dilutions of trace-labeled ^{125}I α Pro 15. The hybrid antibody blocked binding of labeled α Pro 15 to the same extent as binding was blocked (50% decrease at one-half dilution of labeled α Pro 15) using an equivalent amount of cold α Pro 15 for preincubation. A HIT antibody derived from an α Pro 13 hybridoma fusion did not block the binding of trace-labeled α Pro 15, as would be expected since the parental α Pro 13 and α Pro 15 MABs are believed to recognize different epitopes on

TABLE 1.—HIT-mediated depression of protein synthesis in PC-3 prostatic carcinoma cells after 50 hrs of exposure to graded concentrations of HIT and RAC*

Treatment†	% control protein synthesis remaining
Control	100
10^{-10} M RAC	89
10^{-9} M RAC	90
20 µg of α Pro 15 + 10 mM NH_4Cl + 10^{-9} M RAC	68
20 µg of α Pro 15 + 10 mM NH_4Cl + 10^{-10} M RAC	62
20 µg of HIT^1 (2.5 µg of functional hybrid antibody or 0.0125 µg/µl of culture medium)	64
10 µg of HIT^1	84
5 µg of HIT^1	80
20 µg of HIT^1 + 10^{-10} M RAC	66
10 µg of HIT^1 + 10^{-10} M RAC	92
5 µg of HIT^1 + 10^{-10} M RAC	88
20 µg of HIT^1 + 10^{-9} M RAC	32
10 µg of HIT^1 + 10^{-9} M RAC	63
5 µg of HIT^1 + 10^{-9} M RAC	100
20 µg of HIT^1 + 10^{-9} M RAC + 10 mM NH_4Cl	9
10 µg of HIT^1 + 10^{-9} M RAC + 10 mM NH_4Cl	31
5 µg of HIT^1 + 10^{-9} M RAC + 10 mM NH_4Cl	44
20 µg of HIT^2	100
20 µg of HIT^2 + 10^{-9} M RAC	55
20 µg of HIT^3	80
20 µg of HIT^3 + 10^{-9} M RAC	41
20 µg of HIT^4	82
20 µg of HIT^4 + 10^{-9} M RAC	36

* PC-3 cells growing in 96-well microtiter plates were exposed to isolated IgG mixture containing HIT from hybridoma H33IG8D8B6 at concentrations of 20, 10, and 5 µg/200 µl of culture medium/well or to 3 different clonal derivatives of H33IG8D8B6 at a concentration of 20 µg/200 µl only. Cells exposed to HIT at these concentrations were exposed to RAC at concentrations of 10^{-9} and 10^{-10} M. It is apparent that parental α Pro 15 MAB does not depress cellular protein synthesis in conjunction with RAC. Cytotoxic effect of HIT-RAC conjugates was markedly enhanced in the presence of 10 mM NH_4Cl.

†HIT^1 = H33IG8D8B6; HIT^2 = H33IG8D8G7; HIT^3 = H33IG8D8G9; and HIT^4 = H33IG8D8H12.

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[5] Adsorption curves generated by the above experimental approach may appear anomalous, depending on the relative pool size of parental α Pro 15 and anti-RAC monoclonal antibodies and functional and nonfunctional hybrid antibodies. Theoretically, the HIT monoclonal antibody population should consist of $\frac{1}{16}$ of α Pro 15 MABs, $\frac{1}{16}$ of anti-RAC MABs, $\frac{2}{16}$ of functional hybrid antibodies, and $\frac{12}{16}$ of nonfunctional hybrid antibodies. However, shifts in the antibody population may occur as a result of differential production of α Pro 15 heavy and light chains vs anti-RAC heavy and light chains. If one assumes the typical situation of $\frac{2}{16}$ of parental MABs ($\frac{1}{16}$ of α Pro 15 + $\frac{1}{16}$ of RAC) and $\frac{2}{16}$ of functional recombinant HIT, then of an initial 100 µg of MAB population, $\frac{3}{16}$ (18.75 µg) will be available to bind to PC-3 cells; adsorption with RAC will remove $\frac{2}{3}$, or 12.5 µg, of MAB able to bind to PC-3 cells. The same reduction would be applicable to MAB available to bind to RAC after adsorption with PC-3 cells. However, if a theoretical distribution of $\frac{3}{16}$ of parental anti-RAC, $\frac{1}{16}$ of parental α Pro 15, and $\frac{1}{16}$ of functional HITs exists, then $\frac{2}{16}$ (12.5 µg) of the totalantibody population will be able to bind to PC-3; adsorption with PC-3 would remove $\frac{1}{2}$, or 6.25 µg, of the MAB population able to bind to PC-3. Conversely, adsorption of the MAB population with PC-3 would remove only 25%, or 3.12 µg, of antibody available to bind to RAC. It should also be noted that the data in fig 5 were generated using cellular binding to PC-3 and solid-phase assay as a measure of binding to RAC.

the p40 molecule (K. S. Webb et al, unpublished observations).

BIOLOGIC IMPACT OF HIT-RAC CONJUGATES

Since RAC acts at the ribosomal level to inhibit cellular protein synthesis (15), inhibition of ^{14}C-amino acid uptake is a reliable indicator of the effect of HIT-RAC conjugates on viability of cultured prostatic carcinoma cells. The data in figure 3 show that HIT plus RAC depresses ^{14}C-amino acid uptake by PC-3 cells to a level approximately 50% of that obtained in the presence of hybrid antibody alone after 16 hours of exposure. The addition of 10 mM NH_4Cl to the HIT-RAC conjugate caused a further 50% reduction in protein synthesis. The data in table 1 show the effect of variable concentrations of different clonal derivatives of HIT-RAC conjugates on protein synthesis of cultured prostate carcinoma PC-3 cells in the presence and absence of the lysosomotropic agent, NH_4Cl (2,8). The experiment shown in table 1 has been repeated five times using the same H33IG8D8B6 HIT-RAC conjugate or different clonal derivatives thereof and using either PC-3 or DU-145 (18) prostate carcinoma cell lines or epithelial cells originated from a primary prostate tu-

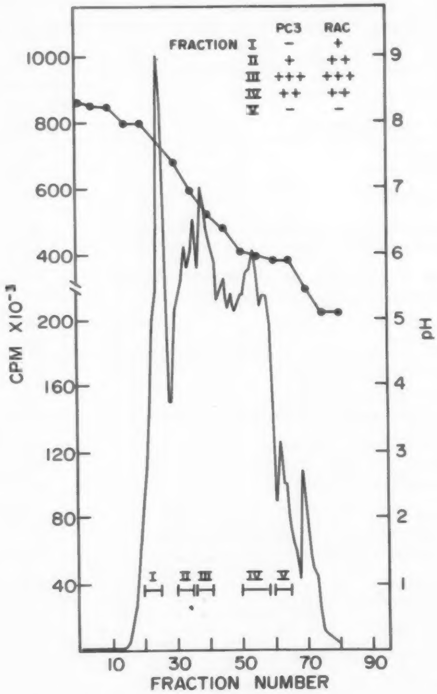

FIGURE 4.—Total IgG, containing HIT antibody, was isolated by Sepharose-protein A (Pharmacia) chromatography, iodinated, and subjected to chromatofocusing over a pH 5-8 gradient. Start buffer consisted of 0.025 M Tris-acetate, pH 8.3. Elution buffer consisted of 74/96 of Polybuffer mixture adjusted to pH 5 with acetic acid. Fractions (1.5-ml) from peak areas (I-V) of the chromatogram were pooled and dialyzed vs PBS containing 2.5% Aquacide (Calbiochem, San Diego, CA) to remove Polybuffer and to concentrate the pooled fractions. Each of the 5 fractions was normalized on a cpm basis and evaluated for binding over a log of dilution to PC-3 cells and to solid-phase RAC. Insert (upper right corner) qualitatively summarizes binding differential exhibited by chromatofocused fractions.

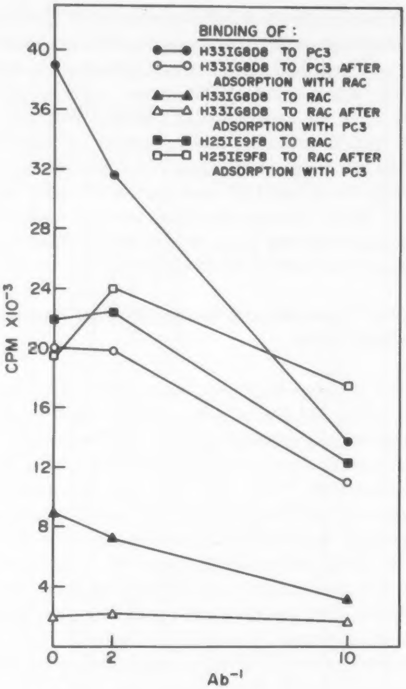

FIGURE 5.—Cross-adsorption experiment demonstrating that binding of IgG isolated from HIT hybridoma culture is quantitatively reduced by prior adsorption with solid-phase RAC. The reciprocal cross-adsorption experiment indicates that binding of IgG to RAC can also be reduced by prior adsorption of the antibody mixture with PC-3 cells. The experiment was conducted by equalizing the HIT IgG and the H25IE9F8 IgG to a neat concentration of 2 mg/ml. The HIT antibody was exposed to a solid-phase RAC plate in triplicate wells at undiluted, 1:2, and 1:10 dilutions. After 90 mins of incubation, antibody was removed from each well of the solid-phase RAC plate and quantitatively transferred to corresponding wells of a microtiter plate containing PC-3 cells and allowed to further equilibrate for 90 mins. Bound antibody was detected with anti-mouse Ig and iodinated protein A. Reciprocal experiment consisted of primary binding to PC-3 cells and quantitative transfer of unbound antibody to a solid-phase RAC plate. Binding of MAB raised against RAC was included as an adsorption control. It is apparent that binding of anti-RAC MAB was quantitatively identical under conditions where MAB was 1st incubated with PC-3 cells and then transferred to solid-phase RAC plate (□———□) or was equilibrated (60 mins, room temperature) with solid-phase RAC microtiter plate without prior incubation with PC-3 cells (■———■). SE did not exceed 10% for any given experimental point.

mor; the results generated were essentially the same as shown in table 1. The biologic impact of the H33IG8D8B6 HIT-RAC conjugate is significant in that cellular protein synthesis is reduced to 32% of control levels at a HIT-RAC dose of 0.1 µg/µl of culture supernatant. Radiolabeled amino acid uptake is reduced to approximately 10% of the control level in the presence of 10 mM NH_4Cl. The hybrid antibody preparation itself reduces protein synthesis to 66% of control levels (table 1). However, the presumed cytotoxic effect of HIT antibody alone is not reproducible; the antibody alone may have a slight effect on protein synthesis; the effect may be transient and disappear at later time points; or HIT antibody alone may exert no effect at all (table 1). In contrast, HIT-RAC conjugates invariably reduce cellular protein to at least 50% of the control level after 20-50 hours of exposure of cells to the immunotoxin.

The data in figure 6 show that various clonal derivatives of HIT-RAC conjugates can discriminate between a target antigen-positive prostate carcinoma (22) cell line and a target antigen-negative pancreatic carcinoma cell line, HPAF-2 (11). After 40 hours of exposure, inhibition of ^{14}C-amino acid uptake in prostate carcinoma cells ranges from 35% to 71% of the uptake level in the presence of hybrid antibody alone. In contrast, none of the three different clonal derivatives of HIT-RAC conjugates exhibited a significant inhibition of radiolabeled amino acid uptake by pancreatic carcinoma cells.

The data in figure 7 show that the biologic impact of HIT-RAC conjugates is not limited to prostate carcinoma cell lines. These HIT conjugates exert a significant effect on protein synthesis of epithelial cells originating from a primary prostatic carcinoma specimen in the presence of 25 µg (0.125 µg/µl of culture medium) of HIT-RAC conjugate; protein synthesis in treated cells was reduced to approximately 25% of control protein synthesis. It is also of note that hybrid antibody alone had no effect on protein synthesis in this experiment.

DISCUSSION

We have presented evidence for the generation of a hy-

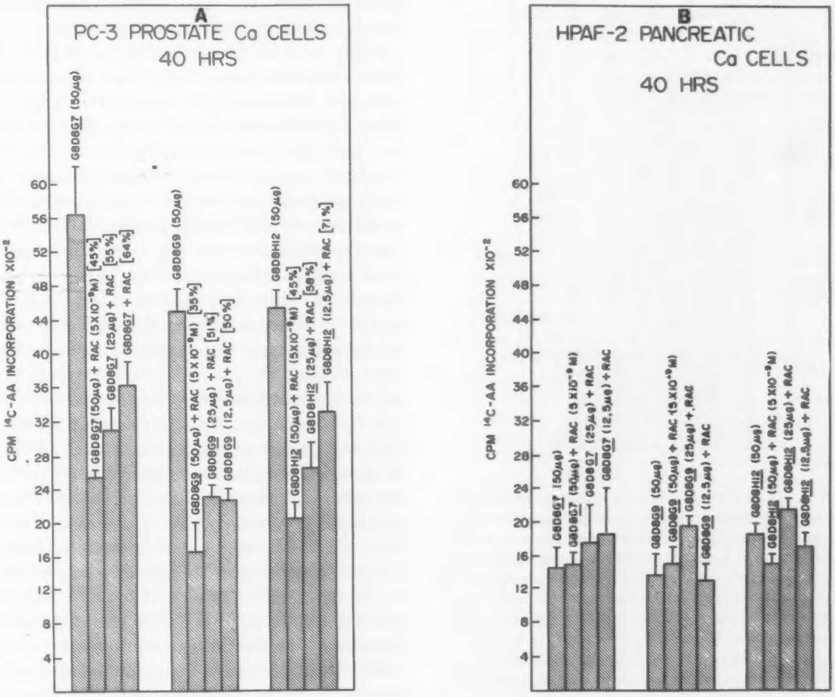

FIGURE 6.—Selective cytotoxic effect of HITs produced by 3 clonal derivatives of H33IG8D8 on PC-3 prostatic carcinoma cells (A) vs p40 antigen-negative HPAF-2 pancreatic carcinoma cells (B). It is apparent that the HIT secreted by each clone depressed protein synthesis in prostatic carcinoma cells to 45%-64% (G7 clone), 33%-50% (G9 clone), and 45%-71% (H12 clone) as compared to the control level of protein synthesis. Control consisted of cells exposed to HIT alone at the highest concentration of IgG used in the experiment. Doses of IgG containing HIT ranged from 50 to 12.5 µg/200 µl of culture medium. Depression of label uptake is, in general, HIT dose-dependent for prostatic carcinoma cells. Uptake of ^{14}C-amino acids by HPAF-2 pancreatic carcinoma cells is unaffected by the same experimental conditions that caused a marked reduction in label uptake by prostate carcinoma cells.

brid antibody with specificity for both the RAC and a prostate-restricted antigen and have demonstrated that the HIT exerts a selective biologic impact on prostate carcinoma cells.

Although we have employed the crude IgG population, encompassing MAB of both parental types, nonfunctional recombinants, and functional hybrid MAB, it is nevertheless apparent that the HIT present in the antibody mixture is capable of exerting a significant biologic impact on prostate carcinoma cells expressing the target antigen. The second-generation HIT hybridoma offers the advantage of synthesizing hybrid antibody in unlimited amounts. Although HIT is produced as a component of a defined mixture of MABs, the hybrid MAB can be easily and reproducibly purified by chromatofocusing methodology. The second-generation hybridomas synthesizing HIT are stable for at least 1 month and possibly longer; it has been our practice to routinely reclone at 4-6-week intervals, although this maneuver may be unnecessary. The HIT offers the advantage of achieving uniform conjugation from preparation to preparation, whereas the number of RACs coupled per immunoglobulin molecule for any given coupling procedure is an inherent variable in chemical coupling methods (2). Also, since the hybrid monoclonal antibody combines with toxin via routine antigen-antibody interaction, alternative therapeutic modalities by which the hybrid antibody may be employed are possible. HIT may be administered in vivo with RAC attached to the antigen-combining site. Alternatively, since RAC is nontoxic unless combined with a moiety (eg, antibody or natural B binding subunit) by which the A chain can exert a biologic effect, it would be possible to temporally segregate administration of hybrid antibody and RAC. The clinical applicability of HIT-RAC conjugates will obviously be contingent upon the in vivo stability of the RAC-hybrid antibody complex.

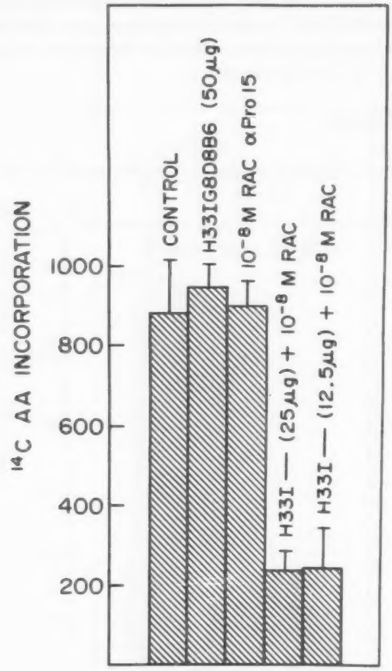

FIGURE 7.—Effect of HIT on prostate carcinoma cells derived from primary surgical prostate carcinoma specimen. Cells were initiated in culture by mincing primary tumor tissue and incubating tissue fragments in 0.1% collagenase for 6 hrs. Resulting cell suspension was diluted into RPMI medium supplemented with NuSerum (Collaborative Research, Inc, Lexington, MA) and plated in Petri dishes precoated with 0.02% gelatin. Microscopic examination at this time indicated that culture was epithelial; no fibroblastic growth was evident. Cells were seeded into 96-well microtiter plates at a density of 5 × 10^4 cells/well. The following day, the cells were exposed to isolated IgG containing HIT and RAC. Controls included cells exposed to RAC alone and to parental α Pro 15 antibody + RAC. Cells were assayed for incorporation of ^{14}C-amino acids into TCA-insoluble protein after 20 and 40 hrs of HIT exposure. The data in fig 7 demonstrate that HIT preparations can effect protein synthesis in cells derived from surgical prostate carcinoma specimens. HIT-induced depression of protein synthesis was approximately 25% of that exhibited by control cells after 40 hrs of exposure to HIT.

Aside from the clinical potential of HIT, the HIT presents a model divergent from that of conventional immunotoxins conjugated by chemical coupling procedures. First, the HIT must, by definition, bind to the cell surface in a univalent mode; this situation is distinct from conventional immunotoxins chemically coupled in a position removed from the antigen-binding site, which usually participate in divalent binding. Cobbold and Waldman (3) have recently stressed the advantages of MABS that have been modified to ensure a univalent binding mode. Specifically, univalent binding allows the attainment of a higher antibody density at the cell surface than that attained with antibodies participating in divalent binding. Also, MABs that are restricted to univalent binding do not induce modulation by extensive rearrangement of cell surface components. Although it is our experience that modulation (ie, internalization of the immune complex) may proceed independently of extensive cell surface antigen redistribution (22), it is conceivable that univalent binding would lessen the chances of modulation by shedding the antigen-antibody complex from the tumor cell surface. Internalization of the immune complex would be beneficial in an immunotherapeutic context, whereas shedding of the complex would thwart immunotherapeutic approaches. In confirmation of the work of Cobbold and Waldman (3), we have observed that radioiodinated HIT can "load" at the cell surface in amounts threefold to fourfold greater than the parental Pro 15 MAB (K. S. Webb, unpublished observations); the immunotherapeutic benefit of higher cell surface saturation densities of immunotoxins is obvious. Second, the HIT model stipulates that the ricin A molecule is closely juxtaposed to the tumor cell surface via interaction of the RAC molecule with the antigen-combining site of the hybrid antibody. In light of the experiments of Ghose et al (6) showing that certain drug-immunoglobulin complexes do not require cellular uptake to exert a cytocidal effect, even in the case where the drug has been shown to interact specifically with DNA, determination of the mechanisms by which HIT-RAC conjugates exert their presumptive cytocidal effect is highly relevant to the future therapeutic use of these conjugates. Our results have shown that the lysosomotropic agent NH_4Cl markedly enhances the biologic effect of HIT-RAC conjugates; these results would suggest internalization of the conjugate (2), although the mechanism of lysosomotropic agents has yet to be fully elucidated. However, in contradistinction to lysosomotropic augmentation, we have not been able to demonstrate internalization (22) of the iodinated HIT-RAC conjugate or internalization of hybrid antibody with only the RAC moiety labeled. Work on the mechanism of action of hybrid MAB-RAC conjugates is currently in progress in our laboratory.

Using a crude HIT preparation, the time required to achieve a 50% depression in protein synthesis compares favorably with chemically conjugated immunotoxins prepared by other investigators (8,20). Wiels et al (25) reported a 50% inhibition of protein synthesis within a 3-hour period using a chemically coupled RAC immunotoxin directed to an antigen on Burkitt's lymphoma cells. Fast kinetics are generally the exception rather than the rule for most immunotoxins generated to date. In general, the slow kinetics exhibited by immunotoxins translate into the necessity for maintaining a high blood level of the immunotoxin for a prolonged time to achieve a therapeutic response. Immunotoxins generated using intact ricin possess a definite advantage over those generated with RAC. Using an intact ricin-IT, Stong et al (19) have recently reported on a > 99.99% kill of leukemia cells. Intact ricin would appear to enter the cell by a pathway separate from RAC and to be transported more efficiently ic than is RAC (8,20). However, the work of Wiels et al (25) with regard to the rapid rate of internalization of a RAC-immunotoxin encourages further investigation into alternative modes of RAC entry into the cell. The HIT described in this report has potential in contributing to such investigations.

We have relied primarily on depression of incorporation of radiolabeled amino acids into cellular protein to evaluate the biologic impact of HIT-RAC conjugates. Although effect of immunotoxins on protein synthesis is a widely accepted method of evaluating the efficacy of immunotoxins (1,7,16,20), bioassays such as inhibition of colony formation should also be used to evaluate actual cytocidal effect.

Last, it should be stressed that in light of known tumor heterogeneity and progression, an immunotoxin directed to a single tumor-restricted surface component is unlikely to be of significant therapeutic value. Therapeutic trials are more likely to be successful if a monolithic approach is not adhered to. Combinations of the same molecule, different epitope, and different molecule-directed immunotoxins must be judiciously evaluated in conjunction with chemotherapeutic protocols, in addition to active nonspecific manipulation of the host immune status.

REFERENCES

1. BUMOL TF, WANG QC, REISFELD RA, ET AL. Monoclonal antibody and an antibody-toxin conjugate to a cell surface proteoglycan of melanoma cells suppress in vivo tumor growth. Proc Natl Acad Sci USA 80:529-533, 1983.
2. CASELLAS P, BROWN JP, GROS O, ET AL. Human melanoma cells can be killed in vitro by an immunotoxin specific for melanoma-associated antigen p97. Int J Cancer 30:437-443, 1982.
3. COBBOLD SP, and WALDMAN H. Therapeutic potential of monovalent monoclonal antibodies. Nature 308:460-462, 1984.
4. COTTON RGH, and MILSTEIN C. Fusion of two immunoglobulin-producing myeloma cells. Nature 244:42-43, 1973.
5. EHRLICH P. A general review of the recent work in immunity. *In* Collected Papers of Paul Ehrlich (Himmelweit F, ed). London, Pergamon Press, 1956, pp 442-447.
6. GHOSE T, GUCLU A, RAMAN R, ET AL. Inhibition of a mouse hepatoma by the alkylating agent Trenimon linked to immunoglobulins. Cancer Immunol Immunother 13:185-189, 1982.
7. GILLILAND DG, STEPLEWSKI Z, COLLIER RJ, ET AL. Antibody-directed cytotoxic agents: use of monoclonal antibody to direct the action of toxin A chains to colorectal carcinoma cells. Proc Natl Acad Sci USA 77:4539-4543, 1980.
8. JANSEN FK, BLYTHMAN HE, CARRIERE D, ET AL. Immunotoxins: hybrid molecules combining high specificity and potent cytotoxicity. Immunol Rev 62:185-216, 1982.
9. KAIGHN ME, NARAYAN KS, OHNUKI Y, ET AL. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest Urol 17:16-23, 1979.
10. MATHE G, LOC TB, and BERNARD J. Effect sur la leucemie 1210 de la souris d'une combinaison par diazotation d'A-methoplerine et de y-globulines de hamsters porteurs de cette leucemie par heterogreffe. Acad Sci (Paris) 246:1626-1628, 1958.
11. METZGAR RS, GAILLARD MT, LEVINE SJ, ET AL. Antigens of human pancreatic adenocarcinoma cells defined by murine monoclonal antibodies. Cancer Res 42:601-608, 1982.
12. MEYERS CD, THORPE PE, ROSS WCJ, ET AL. An immunotoxin with therapeutic potential in T cell leukemia: WTI-ricin A. Blood 65:1178-1185, 1984.
13. NEVILLE DM, and YOULE RJ. Monoclonal antibody-ricin or ricin A chain hybrids. Kinetic analysis of cell killing for tumor therapy. Immunol Rev 62:75-91, 1982.
14. OI VT, and HERTZENBERG LA. Immunoglobulin producing hybrid cell lines. *In* Selected Methods in Cellular Immunology (Mishell B, and Shiigi S, eds). Oxford, W. H. Freeman, 1980, p 351.
15. OLSNES S, FERNANDEZ-PUENTES C, CARRASCO L, ET AL. Ribosome inactivation by the toxic lectins abrin and ricin. Kinetics of the enzymatic activity of the toxic A-chains. Eur J Biochem 60:281-288, 1975.
16. RASO V, and GRIFFIN T. Specific cytotoxicity of a human immunoglobulin-directed F(ab)'-ricin A chain conjugate. J Immunol 125:2610-2626, 1980.
17. RASO V, and GRIFFIN T. Hybrid antibodies with dual specificity for the delivery of ricin to immunoglobulin-bearing target cells. Cancer Res 41:2073-2078, 1981.
18. STONE KR, MICKEY DD, WUNDERLI H, ET AL. Isolation of a human prostate carcinoma line (DU145). Int J Cancer 21:274-281, 1978.
19. STONG RC, YOULE RJ, and VALLERA DA. Elimination of clonogenic

T-leukemic cells from human bone marrow using anti-Mr 65,000 protein immunotoxins. Cancer Res 44:3000-3006, 1984.

20. VITETTA ES, KROLICK KA, MIYAMA-INABA M, ET AL. Immunotoxins: a new approach to cancer therapy. Science 219:644-650, 1983.
21. WARE JL, PAULSON DF, PARKS SF, ET AL. Production of a monoclonal antibody α Pro 3 recognizing a human prostatic carcinoma antigen. Cancer Res 42:1215-1222, 1982.
22. WEBB KS, WARE JL, PARKS SF, ET AL. Monoclonal antibodies to different epitopes on a prostate tumor-associated antigen: implications for immunotherapy. Cancer Immunol Immunother 14:155-166, 1983.
23. WEBB SK, PAULSON DF, PARKS SF, ET AL. Characterization of prostate-tissue-directed monoclonal antibody α Pro 13. Cancer Immunol Immunother 17:7-17, 1984.
24. WEBB KS, ZEIDMAN SM, and WARE JL. Production of murine monoclonal antibodies against ricin A chain. Fed Proc 43:1956, 1984.
25. WIELS J, JUNQUA S, DUJARDIN P, ET AL. Properties of immunotoxins against a glycolipid antigen associated with Burkitts lymphoma. Cancer Res 44:129-133, 1984.
26. YOULE R, and NEVILLE DM. Kinetics of protein synthesis inactivation by ricin-anti-Thy 1.1 monoclonal antibody hybrids. Role of the ricin B subunit demonstrated by reconstitution. J Biol Chem 257: 1598-1601, 1982.

Promotion of Daunorubicin Uptake and Toxicity by the Calcium Antagonist Tiapamil and Its Analogs[1,2]

David Kessel* and Cynthia Wilberding[3]

Studies were performed with the anthracycline-responsive P388 murine leukemia cell line, and with P388/ADR, a subline selected for doxorubicin resistance. In the P388/ADR cell line, the observed 100-fold daunorubicin resistance is associated with an outward drug transport system which can be inhibited by several calcium antagonists, eg, verapamil and nifedipine. An examination of compounds related to tiapamil, a verapamil analog, has identified a structure which is tenfold more effective than verapamil at potentiating anthracycline accumulation and responsiveness in P388/ADR cells. At no nontoxic level of any calcium antagonist examined was it possible to wholly reverse the degree of daunorubicin resistance found in the P388/ADR cell line. [Cancer Treat Rep 69:673–676, 1985]

Resistance to the anthracyclines and certain other drugs in the doxorubicin-resistant P388/ADR subline of the P388 murine leukemia (1–4), and in other drug-resistant cell lines (5–8), is associated with an outward transport process that limits drug accumulation to sublethal levels. This outward transport system exhibits both an energy requirement (3,7–10) and structural specificity (9,11). Verapamil (12–15) and other calcium antagonists (14) can promote responsiveness to anthracyclines and vinca alkaloids in drug-resistant cell lines, apparently via inhibition of this outward anthracycline transport system. This phenomenon is not associated with alterations in calcium fluxes (16), and, in the case of verapamil, derives from competition for the outward transport process (17). Clinical use of drug combinations containing verapamil may, however, be limited by the potentially toxic concentrations (2–6 μM) required for circumvention of anthracycline resistance in vitro (12–15). In this study, we examined a series of compounds structurally related to tiapamil (18–21), a sulfur-containing analog of verapamil. A potent anthracycline exodus inhibitor was thereby identified.

MATERIALS AND METHODS

The doxorubicin-resistant P388/ADR cell line was derived from the P388 murine leukemia; drug responsiveness patterns of both cell lines have been described (4,22). P388 and P388/ADR cells were maintained in cell culture using Fischer's medium (Gibco, Grand Island, NY) supplemented with 10% horse serum and 1 μM mercaptoethanol. [14-^{14}C]Daunorubicin (31 Ci/mol) was obtained from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD. Purity was > 98% by thin-layer chromatographic assay. Verapamil was obtained from Knoll Pharmaceutical Co, Whippany, NJ; tiapamil and its analogs were obtained from Drs. H. Neumann and H. Ramuz, Hoffmann-LaRoche and Co, Basel, Switzerland.

Uptake studies were carried out at cell densities of 2×10^6/ml (7 mg/ml, wet weight) in HEPES-buffered Fischer's medium at pH 7.2. The initial DNR level was 0.1 μM, approximately 7000 counts/min/ml. In no experiment was > 20% of total radioactivity accumulated by cells. Incubations were terminated by centrifugation (30 secs, 200 × *g*) and the cells were washed once with 150 mM NaCl at 0°C. Effects of verapamil, tiapamil, and analogs of the latter were measured by adding these agents 5 mins before the labeled DNR. Accumulation of radioactivity was assessed by liquid scintillation counting.

For studies of outward DNR transport, cells were loaded with 2 μM (P388) or 10 μM (P388/ADR) labeled DNR for 10 mins at 37°C, so that the initial intracellular drug concentrations in P388 and P388/ADR cells were similar (30 pmols/10^6 cells). The drug-loaded cells were

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[3] Departments of Medicine and Pharmacology, Wayne State University School of Medicine, and Harper-Grace Hospitals, Detroit, MI.

**Reprint requests to:* David Kessel, Department of Medicine, Harper-Grace Hospitals, Detroit, MI 48201.

then suspended in fresh growth medium at 37°C, and effects of calcium antagonists on efflux were measured as described (27).

Cytotoxicity studies in vitro were carried out by treating P388 and P388/ADR cell cultures for 4 hours with DNR plus other agents as specified. After exposure to daunorubicin, the cells were then washed and diluted appropriately into growth medium plus 0.2% agar. Colonies of control versus treated cells were counted after 7 days. Cytotoxicity of verapamil, tiapamil, and analogs of the latter analogs were also measured by this procedure.

RESULTS

P388 cells incubated in medium containing 0.1 μM DNR contained approximately 5 pmols of drug/10^6 cells after 30 mins. Kinetic studies indicated that a steady-state of drug accumulation was reached at this time. The corresponding DNR level in P388/ADR cells was 0.8 pmol/10^6 cells. Uptake of 0.35 pmol/10^6 cells represents a cell/medium distribution ratio of one. Since cytotoxicity studies on P388/ADR were carried out using a DNR concentration of 0.8 μM DNR, we examined the effect of the calcium antagonists on steady-state DNR accumulation using an extracellular DNR level of 1 μM; table 1 shows levels of verapamil, tiapamil, and three tiapamil analogs which doubled the steady-state level of DNR in P388/ADR cells (EC_{100} levels). These drug concentrations increased steady-state DNR levels in P388 cells by < 10%.

We examined a series of 13 analogs of tiapamil containing a variety of functional groups. Four of the most active structures are shown in figure 1 (D = tiapamil). The most effective agent examined was structure A. This agent was tenfold more potent than verapamil at promoting daunorubicin toxicity against P388/ADR cells (table 1). A comparison of the different structures represented indicates that a naphthyl substituent is superior to dimethoxyphenyl for promotion of DNR accumulation, as is a dithiane, rather than a dithiane tetraoxide moiety.

In other studies, we examined drug exodus from cells loaded with DNR. When these cells were resuspended in growth medium, approximately 80% of the DNR was rapidly lost from P388/ADR ($T_{1/2}$ = 1.6 mins). When calcium antagonists were present during exodus studies, at concentrations specified in column 2 of table 1, the half-life of the loosely bound drug pool was increased to 5 ± 1 mins. In contrast, the DNR pool in P388 cells was more tightly bound ($T_{1/2}$ = 60 mins), and DNR exodus was not affected by presence of the calcium antagonists during exodus measurements.

TABLE 1.—Effects of calcium antagonists on daunorubicin transport and toxicity in P388/ADR cells

Antagonist*	EC_{100} (transport)†	LD_{99} (cytotoxicity)‡
Verapamil	2.5	10
A	0.5	1.0
B	2.0	10
C	20	70
D (tiapamil)	150	500

* Structures shown in fig 1.

†Drug level (μM) required to promote the steady-state uptake of daunorubicin by 100%. In 5 experiments, a variation of ± 15% of these Nos. was encountered.

‡Drug level (μM) which, combined with 0.8 μM DNR, results in a 99% cell kill of P388/ADR cells (4-hr exposure). A variation of ± 20% of these values was found in 3 separate experiments.

Cytotoxicity studies showed a correlation between effects of the different tiapamil analogs on DNR exodus versus promotion of DNR toxicity. Under conditions used here, the LD_{99} level of DNR against P388 cells was 0.12 ± 0.02 μM, while the LD_{99} DNR level in P388/ADR was

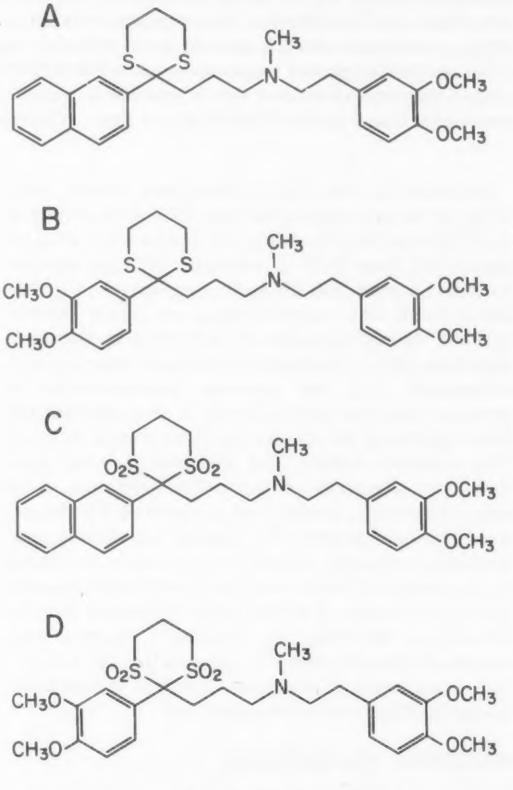

FIGURE 1.—Structures of tiapamil and its analogs. A = *N*-[3,4-dimethoxyphenyl]-2-[2-naphthyl]-*N*-methyl-*m*-dithiane-2-propylamine Ro 11-2933/001; B = *N*-(3,4-dimethoxyphenethyl)-2-(3,4-dimethoxyphenyl)-*N*-methyl-*m*-dithiane-2-propylamine hydrochloride, Ro 10-6852-001-6; C = *N*-(3,4-dimethoxyphenethyl)-*N*-methyl-2-(2-naphthyl)-*m*-dithiane-2-propylamine 1,1,3,3,-tetraoxide hydrochloride, Ro 11-3651/001-3; D = tiapamil, *N*-(3,4,-dimethoxyphenyl)-2-(3,4-dimethoxyphenyl)-*N*-methyl-*m*-dithiane-2-propylamine-1,1,3,3,tetraoxide hydrochloride, Ro 11-1781.

12 ± 1.5 μM. Table 1 shows the levels of tiapamil and its analogs, which, combined with 0.8 μM DNR, kill 99% of P388/ADR cells. In other studies, we found that these concentrations of the calcium antagonists were nontoxic to P388 or P388/ADR cells. The addition of tiapamil or its analogs, at concentrations specified in table 1, did not significantly affect the toxicity of 0.04 μM DNR toward P388 cells. At no tolerated level of tiapamil or its analogs could we achieve an LD_{99} level of toxicity with P388/ADR cells using 0.12 μM DNR.

DISCUSSION

The finding that certain calcium antagonists can promote responsiveness to anthracyclines and other natural products, in certain drug-resistant cell lines (12-15), could have therapeutic implications in the treatment of human neoplastic disease. The clinical usefulness of such combinations may be limited by toxicity considerations. In a report on verapamil pharmacology in man (23), the peak plasma level produced by long-term oral doses of 80 mg every 6 hours was 255 ng/ml (0.6 μM). This level is eightfold lower than the verapamil concentration required to double steady-state DNR accumulation by P388/ADR cells (table 1). Moreover, the verapamil dosages required to promote DNR responsiveness in the tumor-bearing mouse ranged from 25 to 50 mg/kg (12). In man, the usual iv verapamil dose is 0.15 mg/kg (24). Oral ingestion of 2 and 5.6 g of verapamil by adults was accompanied by reversible cardiac toxicity (25,26). It might therefore be feasible to attain plasma levels of verapamil which would potentiate anthracycline responsiveness in cancer patients with drug-resistant tumors.

In this regard, Rogan et al (28) recently demonstrated that verapamil could potentiate doxorubicin-induced toxicity toward human ovarian tumor cell lines in culture. This result suggests a role for anthracycline-verapamil combinations in the therapy of drug-resistant neoplasia. We have identified an analog of tiapamil which was an order of magnitude more potent than verapamil for promotion of DNR toxicity against P388/ADR cells. This effect was associated with a corresponding promotion of DNR accumulation, apparently via inhibition of an enhanced mode of drug exodus which has been associated with several examples of anthracycline resistance (3,7-11). Further studies on the toxicology of Ro 11-2933 will be needed before its role in chemotherapy can be assessed. In preliminary acute toxicity studies in the mouse, we found it to be no more toxic than verapamil.

It is interesting to note that no nontoxic level of any calcium antagonist examined here could wholly reverse the 100-fold level of DNR resistance seen in P388/ADR cells. Such a result suggests additional modes of drug resistance in P388/ADR which are not based on impaired drug accumulation. But impaired drug accumulation appears to be among the first effects associated with developing anthracycline resistance (29), and inhibition of outward anthracycline transport might be helpful in clinical therapy programs. We have recently established that the circumvention of daunorubicin resistance by verapamil involves competition for a common outward drug transport system (17). The structure-activity relationships established in the present study may prove useful in suggesting the design of more effective agents for circumvention of this mode of anthracycline resistance.

REFERENCES

1. INABA M, and JOHNSON R. Decreased retention of actinomycin D as the basis for cross-resistance in anthracycline-resistant sublines of P388 leukemia. Cancer Res 37:4629-4634, 1977.
2. INABA M, and JOHNSON RK. Uptake and retention of adriamycin and daunorubicin by sensitive and anthracycline-resistant sublines of P388 leukemia. Biochem Pharmacol 27:2123-2130, 1978.
3. INABA M, KOBAYASHI H, SAKURAI Y, ET AL. Active efflux of daunorubicin and adriamycin in sensitive and resistant sublines of P388 leukemia. Cancer Res 39:2200-2203, 1979.
4. JOHNSON RK, CHITNIS MP, EMBREY WM, ET AL. In vivo characteristics of resistance and cross-resistance of an adriamycin-resistant subline of P388 leukemia. Cancer Treat Rep 62:1535-1547, 1978.
5. DANØ K. Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells. Biochem Biophys Acta 323:466-473, 1973.
6. KESSEL D, BOTTERILL V, and WODINSKY I. Uptake and retention of daunomycin by mouse leukemic cells as factors in drug response. Cancer Res 28:938-941, 1968.
7. SKOVSGAARD T. Transport and binding of daunorubicin, adriamycin and rubidazone in Ehrlich ascites tumour cells. Biochem Pharmacol 26:215-222, 1977.
8. SKOVSGAARD T. Circumvention of resistance to daunorubicin by *N*-acetyldaunorubicin in Ehrlich ascites tumor. Cancer Res 40: 1077-1083, 1980.
9. SKOVSGAARD T. Carrier-mediated transport of daunorubicin, adriamycin and rubidazone into Ehrlich ascites tumor cells. Biochem Pharmacol 27:1221-1227, 1978.
10. SKOVSGAARD T. Mechanism of cross-resistance between vincristine and daunorubicin in Ehrlich ascites tumor cells. Cancer Res 38: 4722-4727, 1978.
11. CHITNIS MP, and JOHNSON RK. Biochemical parameters of resistance of an adriamycin-resistant subline of P388 leukemia to Emetine, an inhibitor of protein synthesis. JNCI 60:1049-1054, 1978.
12. SLATER LM, MURRAY SL, WETZEL MW, ET AL. Verapamil restoration of daunorubicin responsiveness in daunorubicin-resistant Ehrlich ascites carcinoma. J Clin Invest 70:1131-1134, 1980.
13. TSURUO TO, IIDA H, TSUKAGOSHI S, ET AL. Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res 41:1967-1972, 1981.
14. TSURUO T, IIDA H, TSUKAGOSHI S, ET AL. Increased accumulation of vincristine and adriamycin in drug-resistant P388 tumor cells following incubation with calcium antagonists and calmodulin inhibitors. Cancer Res 42:4730-4733, 1982.
15. TSURUO T, IIDA H, YAMASHIRO M, ET AL. Enhancement of vincristine- and adriamycin-induced cytotoxicity by verapamil in P388 leukemia and its sublines resistant to vincristine and adriamycin. Biochem Pharmacol 31:3138-3140, 1982.
16. KESSEL D. Membrane modification can reverse a mode of anthracycline resistance. Proc Am Assoc Cancer Res 24:252, 1983.
17. KESSEL D, and WILBERDING C. Mode of action of calcium an-

tagonists which alter anthracycline resistance. Biochem Pharmacol 33:1157-1160, 1984.

18. EIGENMANN R, BLABER L, NAKAMURA K, ET AL. Tiapamil, a new calcium antagonist. 1. Demonstration of activity and related studies. Arzneimittelforsch 31:1393-1401, 1981.
19. EIGENMANN R, GEROLD M, HEFTI F, ET AL. Tiapamil, a new calcium antagonist. 2. Further pharmacological characterization. Arzneimittelforsch 31:1401-1410, 1981.
20. RAMUZ H. A new calcium antagonist, Ro-11-1781, and its metabolites. Arzneimittelforsch 28:2048-2051, 1978.
21. RAMUZ H. Tiapamil, Ro 11-1781, a Ca^{++} antagonist. An improved synthesis, physicochemical properties, preparation of a deuterated derivative. Cardiology 69(suppl 1):26-30, 1982.
22. JOHNSON RK, OVEJERA AA, and GOLDIN A. Activity of anthracyclines against an adriamycin (NSC-123127)-resistant subline of P388 leukemia with special emphasis on cinerubin A (NSC-18334). Cancer Treat Rep 60:99-102, 1976.
23. FREEDMAN SB, RICHMOND DR, ASHLEY JJ, ET AL. Verapamil kinetics in normal subjects and patients with coronary artery spasm. Clin Pharmacol Ther 30:644-665, 1981.
24. MCGOON MD, VLIETSTRA RE, HOLMES DR, JR, ET AL. The clinical use of verapamil. Mayo Clin Proc 57, 495-510, 1982.
25. CANDELL J, VALLE V, SOLER M, ET AL. Acute intoxication with verapamil. Chest 75:200-201, 1979.
26. DEFAIRE U, and LUNDMAN T. Attempted suicide with verapamil. J Cardiol 6:195-198, 1977.
27. KESSEL D, and WHEELER C. *m*-AMSA as a probe for transport phenomena associated with anthracycline resistance. Biochem Pharmacol 33:991-993, 1984.
28. ROGAN AM, HAMILTON TC, YOUNG RC, ET AL. Reversal of adriamycin resistance in human ovarian cancer. Science 244: 994-996, 1984.
29. WHEELER C, RADER R, and KESSEL D. Membrane alterations associated with progressive adriamycin resistance. Biochem Pharmacol 31:2691-2693, 1982.

Increased Glutathione-*S*-Transferase Activity in a Cell Line With Acquired Resistance to Nitrogen Mustards[1]

Ann L. Wang[2,3] and Kenneth D. Tew[2,*]

A Walker 256 rat mammary carcinoma cell line (WR) resistant to bifunctional nitrogen mustards has been shown to have an approximate twofold increase in bulk glutathione-*S*-transferase activity compared to the parent cell line. Substrate specificity studies suggest that higher levels of Y_b subunit contribute to the increased activity. By exposing WR cells to additional chlorambucil, either as a single concentration (50 μg/ml) or at 5 μg/ml for 10 days, transferase activity was further increased by up to three times the normal WR level. By using colony-forming assays, mitotic index depression, or trypan blue exclusion, the increased transferase activity could be correlated with an increase in resistance of these cells to either subsequent chlorambucil or a different bifunctional nitrogen mustard, phosphoramide mustard. [Cancer Treat Rep 69:677–682, 1985]

The phenotypic expression of acquired resistance to antitumor drugs is frequently the primary cause of therapeutic failures in cancer patients. Because tumor cells possess a high degree of population heterogeneity and a rapid cell division rate, there is great potential for selection of resistant populations through treatment protocols which deliver sublethal concentrations of drug. In the case of bifunctional nitrogen mustards, there exists evidence of impaired drug uptake (1) or increased glutathione levels (2,3) as mechanisms of resistance in some tumor cells. The glutathione-*S*-transferase isozymes have been studied in detail with respect to their role in detoxification of a variety of xenobiotics (4–6). Molecular heterogeneity of these isozymes allows for the conjugation of a diverse range of toxic electrophiles with glutathione. The electrophilicity of some mustard metabolites is comparatively high, with the result that their direct interaction with reduced glutathione is kinetically favorable. However, the glutathione-*S*-transferase enzymes can still contribute to the detoxification process by increasing the speed and efficiency of such conjugation reactions. The greater efficiency may be a factor in determining whether active alkylating species from the mustards reach a critical intracellular threshold level and interact with important cellular nucleophiles or are effectively detoxified before such damage is induced. A Walker 256 rat mammary carcinoma cell line (WR) resistant to bifunctional nitrogen mustards (7) has been shown to possess an aberrant glutathione reductase activity (8), a determinant of collateral sensitivity to nitrosoureas. This cell line expresses an approximate 20-fold acquired resistance to nitrogen mustards. Because of the existing data showing that glutathione-*S*-transferase activity is inducible in liver following exposure to sublethal concentrations of electrophilic drugs (9–12), this study considers a possible role of this family of enzymes in determining acquired resistance in the WR cell line.

MATERIALS AND METHODS

Cell cultures.—WR cell lines were obtained as previously described (7). The WR cells, which possess 15–20-fold resistance to alkylating agents, were selected from the parent cell line following exposure to chlorambucil (13). Additionally, these cells exhibit no cross-resistance to nitrosoureas (7). The cells are maintained in static suspension in Dulbecco's minimum essential media (4.5 g/L of glucose; M. A. Bioproducts, Walkersville, MD) and in 10% heat-inactivated fetal calf serum (KC Biologicals, Kansas City, KS) in a 5% CO_2 atmosphere. Resistance is maintained by biannual

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[2] Laboratory of Molecular Pharmacology, Division of Medical Oncology, Vincent T. Lombardi Cancer Research Center, Georgetown University, Washington, DC.

[3] *Present address:* Molecular Pharmacology Department, Smith Kline and French Laboratories, Swedeland, PA.

[*] *Reprint requests to:* Kenneth D. Tew, PhD, Laboratory of Molecular Pharmacology, Division of Medical Oncology, Vincent T. Lombardi Cancer Research Center, Georgetown University, 3800 Reservoir Rd, Washington, DC 20007.

exposure to chlorambucil (20 μg/ml).

Glutathione-S-transferase measurements.—Cells were harvested at approximately 4 × 10^5/ml by centrifugation. The cells were then lysed with glass-distilled H_2O with a 30-second sonication on a sonic dismembrator (Artek, Farmingdale, NY) at a power setting of 60 and spun down in an Eppendorf centrifuge at 10,000 × *g*. This procedure was designed to leave microsomal material in the supernatant. Aliquots were then assayed using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (14). In subsequent experiments, activities were obtained following published procedures using the following substrates: *trans*-4-phenyl-3-buten-2-one (14), ethacrynic acid (14), *p*-nitrophenyl acetate (15), and Δ^5-androstene-3,17-dione (16). Protein determinations were made using a Bio-Rad protein assay, with bovine gamma globulin as a standard (Bio-Rad Laboratories, Richmond, CA).

Total cellular glutathione.—Ten milliliters of log-phase cells (approximately 5 × 10^5/ml) were harvested by centrifugation, lysed with distilled H_2O, and the proteins precipitated with a final concentration of 3% sulfosalicylic acid. The cellular glutathione levels were then determined by the method of Griffith (17).

Mitotic index determinations.—Cells were harvested by centrifugation and the visualization of mitotic figures was achieved using a modified differential stain technique (18). Mid-log-phase cells (4 × 10^5/ml) were pelleted and washed three times with 5 ml of 3:1 methanol:acetic acid containing 4 mM $MgCl_2$ and 1.5 mM $CaCl_2$; each wash was for 15 minutes at room temperature. Cells were dropped onto alcohol-cleaned slides and air-dried at 37°C overnight. Resulting smeared slides were placed at 4°C in 5% perchloric acid for 24 hours and subsequently washed in several changes of deionized water. Cells were stained in a filtered solution of 0.5% brilliant blue R (Sigma, St. Louis, MO) and 0.5% Safranin O (Sigma) for 24 hours at room temperature. Slides were exhaustively washed with deionized water, air-dried, and mounted. Cytoplasm was stained light blue and chromatin was stained red. In mitotic figures the spindle was stained purple and was easily discernible.

Colony-forming assays.—Cell survival was determined by a 0.3% agar colony-forming assay following drug treatment as described previously (7). Drug incubations were for 1 hour for colony-forming assays and 2 hours for mitotic index determinations.

Chlorambucil induction.—WR cells were suspended in complete media at a cell concentration of 2 × 10^5 and then treated with either a single dose of drug (50 μg/ml of chlorambucil suspended in absolute ethanol) or multiple (10-day) doses of 5 μg/ml (total, 50). Viability was estimated using trypan blue exclusion and cells were harvested for transferase determinations.

RESULTS

Table 1 reflects the resting thiol status in the WR cell line and in the parent cell line (WS). Reactivity with CDNB reflects the overall, or bulk, glutathione-*S*-transferase activity in the cell lines. Activity is increased approximately twofold in the WR cells. Total reduced glutathione levels were similar. A battery of substrates, alone or in combination (table 1), give information which can be related to the reactivity of specific isozymes (15) and their component subunits (19). WS cells have higher activity with ethacrynic acid and lower activity with Δ^5-androstene-3,17-dione and *p*-nitrophenyl acetate, implying Y_c subunit prevalence. Conversely, these same data suggest an increase in the Y_b subunit in WR. *trans*-4-Phenyl-3-buten-2-one has a high degree of specificity for Y_b, subunit, suggesting that WS has higher levels of this subunit compared to WR.

Tables 2 and 3 show experiments in which WR cells were treated with either a single dose or multiple doses of chlorambucil, as described in the Materials and Methods section. Control cultures of WR cells were maintained concomitantly to allow for simultaneous control transferase estimations. For the single 50-μg/ml concentration (table 2), an initial depletion of activity 1 and 3 days following drug corresponded with a reduction in viability as measured by trypan blue exclusion. At and following Day 5, an increased CDNB reactivity was seen. The initial increases on Days 5, 7, and 10 were associated with a maximum depression in viability. Glutathione-S-transferase activities were measured once viability had recovered to > 95%.

Increased enzyme activity was apparent even at low cell viability. When cultures had recovered to > 95% viable, a twofold to threefold increased glutathione-*S*-transferase was maintained. The multiple-dose experiments (table 3) showed an immediate reduction in ac-

TABLE 1.—Thiol and substrate status of WS and WR cell lines*

	Cell line	
	WS	WR
Thiol		
Total cellular glutathione†	6.25 × 10^{-7}	7.81 × 10^{-7}
Bulk transferase activity CDNB used as the substrate‡	12.7	25.4
Substrate		
trans-4-Phenyl-3-buten-2-one‡	4.0	0.5
Δ^5-Androstene-3,17-dione‡	None detected	0.25
p-Nitrophenyl acetate‡	3.2	5.1
Ethacrynic acid‡	1.6	0.98

*Results are means of at least 4 experiments. SDs are < 10% of the mean.

†mols/mg of protein.

‡nmols/mg/min.

TABLE 2.—Effects of single-dose (50 μg/ml) chlorambucil[*]

Day following drug	Experiment A		Experiment B	
	CDNB activity	Viability	CDNB activity	Viability
0	100	> 95	100	> 95
1	65	80	75	64
3	79	45	65	30
5	125	42	152	27
7	270	< 5	256	< 5
10	343	< 5	216	< 5
14		38		15
16		85		58
17		95		84
21		> 95	200	> 95
22	198	> 95		> 95
24	178	> 95	231	> 95
26	251	> 95	245	> 95
27	172	> 95	312	> 95
28	202	> 95	244	> 95
30	295	> 95	261	> 95
33	370	> 95	397	> 95

[*] All CDNB activity is expressed as % of control (based on control of the series of assays carried out on the same day). Viability determined by trypan blue exclusion.

tivity which was not coincident with a depression in viability. Increased activity was detected by Day 10 (the final treatment day), which was also the point at which the viability of the cells decreased. There was no consistent increase in activity during the 3 weeks posttreatment.

Analysis of other cytosolic and microsomal detoxification enzymes revealed that only trace amounts of total cytochrome P450, UDP-glucuronyl transferase, and epoxide hydrolase existed in both WS and WR cells. Using the same cytosolic fraction (prepared from 1 × 10^9 cells), extremely high levels of the quinone-specific enzyme DT-diaphorase were present. These values, calculated as described previously (20), were 1312 nmols/mg of protein/minute for WS cells and 763 nmols/mg of protein/minute for WR cells.

Established populations of cells from these treatments were exposed to various concentrations of chlorambucil and subsequent viability measurements were carried out. For single-exposure experiments (table 2), Day-21 posttreatment cells were used; for multiple-exposure experiments (table 3), Day-18 posttreatment cells were used. Table 4 shows the mitotic index response to various chlorambucil concentrations. The differential cytotoxicity of chlorambucil in WR and WS cells was demonstrated. Cells pretreated with the sin-

TABLE 3.—Effects of multiple-dose (5 μg/ml) chlorambucil for 10 days[*]

Day following drug	Experiment C		Experiment D	
	CDNB activity	Viability	CDNB activity	Viability
0	100	> 95	100	> 95
1	24	> 95	61	> 95
3	20	> 95	61	90
5	25	92	19	95
10	128	52	206	44
14		15		20
16		11		20
18		73		84
21	114	77		87
22		87	58	> 95
24	85	> 95	71	> 95
26	110	> 95		> 95
27	135	> 95	85	> 95
28	129	> 95	80	> 95
30	151	> 95	138	> 95
33		> 95	143	> 95

[*] On Day 11 the drug-containing media were removed and fresh media were added. CDNB activity is expressed as % of control (based on control for the series of assays carried out on the same day). Viability determined by trypan blue exclusion.

TABLE 4.—Effect of subsequent chlorambucil challenge on the mitotic index of chlorambucil-induced WR cell culture*

Chlorambucil	Cell line					
	WS	WR	WRA	WRB	WRC	WRD
0	5.6	4.8	4.6	4.5	3.9	4.1
25	4.8	4.7	4.8	4.7	4.2	4.0
50	1.9	3.9	4.7	4.4	3.6	3.5
100	0.8	2.2	3.7	3.5	2.9	2.9
250	0	1.6	2.5	2.7	1.4	1.2
500	0	0.2	1.5	1.3	0.4	0.8

* Differential staining was described in the Materials and Methods section. Results based on counts of > 4000 stained cells. Mitotic index calculated as % of cells screened which were in prophase, metaphase, anaphase, or telophase. Designation of cells as WRA, WRB, WRC, and WRD corresponds to experiments in tables 2 and 3.

gle 50-μg/ml dose (WRA and WRB) showed an increased resistance to mitotic index suppression (table 4). Cells exposed to multiple challenge had mitotic index values similar to WR cells. Figure 1 shows colony-forming assay data for WS, WR, WRB, and WRD cells (cf table 2) following a subsequent chlorambucil challenge. WRB cells demonstrated an increased survival which corroborated the mitotic index data. Similarly, WRD cells did not show a significantly different survival from WR, mirroring the mitotic index data in table 4.

In a separate experiment, a different culture of WR cells was exposed to a single 50-μg/ml dose of chlorambucil and allowed to re-establish viability in a 3-week period following drug treatment. At this time, glutathione-*S*-transferase levels were measured at 31.3 ± 1.5 nmols/mg of protein/minute using CDNB as a substrate. A soft-agar colony-forming assay (fig 2) was carried out to test the comparative response of this cell line to phosphoramide mustard. Previous studies (7) have shown that WR cells, when compared to WS cells, exhibit an equivalent resistance to this drug as compared to chlorambucil. Figure 2 shows that this culture exhibited an approximately 20%-30% increased resistance to phosphoramide mustard. This finding is consistent with the approximate 20% increase in glutathione-*S*-transferase activity.

DISCUSSION

In rat liver at least seven glutathione-*S*-transferases have been identified and characterized to varying degrees. They are known to consist of binary combinations of four distinct subunits, Y_a, Y_b, $Y_{b'}$, and Y_c, arranged in homodimeric or heterodimeric forms (10,11). However, the relationship between these enzymes is somewhat obscure. Recent evidence has indicated that these enzymes are unequally distributed among various tissues (22,23). It has also been demonstrated that the subunits are themselves products of separate genes (21,24). Little information is available on tumor cell glutathione-*S*-transferases or their possible role in drug resistance. This present study is an initial report of an increased level of glutathione-*S*-transferase activity in the WR cell line which is known to express an approximate 20-fold resistance to bifunctional nitrogen mustards. The absence of a direct quantitative correlation between the degree of resistance and the increase in enzyme activity is of potential relevance and could suggest that multiple factors contribute to the resistance phenotype of WR cells. Earlier studies (13) have suggested that the inability to raise levels of cyclic adenosine monophosphate-binding protein may contribute to the resistance phenotype properties of the WR cells. However, the cyclic adenosine monophosphate data do not provide a complete quantitative correlation with resistance. Furthermore, the possibility that increased "bulk" transferase activity may be masking an amplification of a nitrogen mustard-specific isozyme is currently under investigation.

Previous studies have shown that nitrosourea toxicity may be influenced by pretreatment of mice with various effectors of hepatic microsomal enzyme activity (25). While liver cells have a variety of detoxification enzymes, we found only trace activities of total cytochrome P450, UDP-glucuronyl transferase, and epoxide hydrolase in the Walker cell lines. A quinone-specific enzyme, DT-diaphorase, was present at much higher activity than in normal liver (20), but was less active in the WR than WS cells, suggesting that it had little relevance to the acquired resistance properties of this cell line.

Treatment of WR cells with chlorambucil concentrations which allow population recovery provided data which suggested that some cells within the heterogeneous tumor population are able to express even higher levels of glutathione-*S*-transferase activity. It has been shown that the glutathione-*S*-transferases are inducible in the cytosolic fraction of rat liver by phenobarbital, *trans*-stilbene oxide, and 3-methylcholanthrene (10). The induction patterns produced by these compounds are variable, with Y_a and Y_c being the most easily induced (10). When extrahepatic tissues were studied following treatment with 3-methylcholanthrene or pheno-

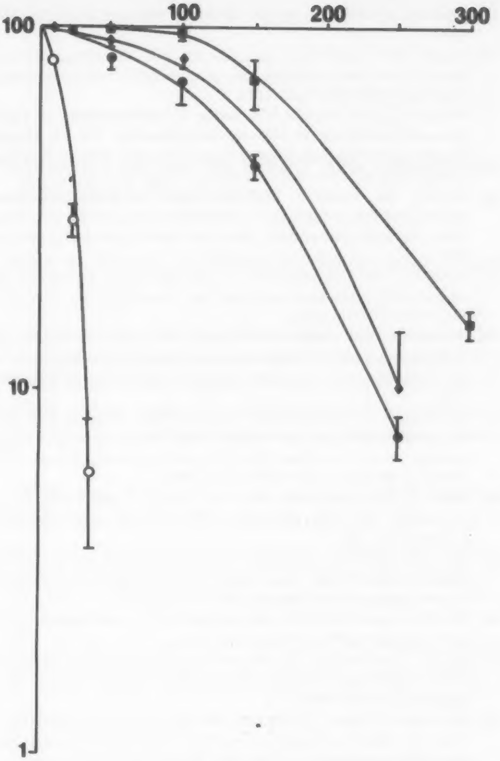

FIGURE 1.—Effect of chlorambucil on colony-forming ability of WS, WR, and selected cell lines WRB and WRD (cf tables 2 and 4). Abscissa is the concentration of chlorambucil in micromolar for 1-hr incubation prior to dilution with 100-fold fresh medium. Ordinate is log scale of colony formation as % of control. Points are ± SD of triplicate experiments. Control plating efficiencies were > 40% for each cell line. ◆ = WRD; ■ = WRB; ● = WR; and ○ = WS.

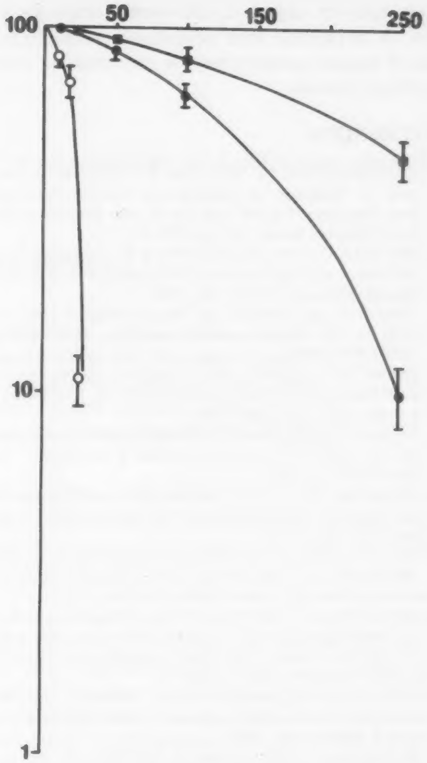

FIGURE 2.—Effect of phosphoramide mustard on colony-forming ability of WS, WR, and selected cell line [chlorambucil (50 μg/ml) survivors; *see* text]. Abscissa is the concentration of phosphoramide mustard in micromolar for 1-hr incubation prior to dilution of drug with 100-fold fresh medium. Ordinate is log scale of colony formation as % of control. Points are means of quadruplicate experiments. Control plating efficiencies were > 40% for each cell line. ■ = selected from WR by single 50-μg/ml exposure of chlorambucil; ● = WR; and ○ = WS.

barbital, no induction of glutathione-*S*-transferase was found (11). This information implies that there is a potential for differential regulation of the glutathione-*S*-transferase subunits. The present data do not distinguish the possible induction of activity [through increased mRNA (26)] or selection of a population with intrinsically higher enzyme activity. Since a large number of cells are killed prior to re-establishment of a viable dividing cell culture (cf tables 2 and 3), the selection of a pre-existing high glutathione-*S*-transferase population is a distinct possibility. Certainly, the repeated lower concentration exposures do not result in higher transferase levels, and this approach should have been more conducive to an induction mechanism. Heterogeneity in the chromosomal complement of WR cells has been reported previously (7,27). This is indicative of a mixed tumor cell population and would be consistent with the concept of selecting subpopulations.

Consideration of these data from a preclinical or clinical standpoint would suggest that suboptimal drug concentrations, when they reach the tumor, may select cells which are more adapted through increased glutathione-*S*-transferase activity to resist a second-drug treatment. Since these data suggest a cross-resistance to phosphoramide mustard, there may be a basis for a more general mechanism of resistance to this class of antitumor compounds. Whether there are qualitative as well as quantitative differences in the glutathione-*S*-transferases of WR cells compared to WS cells and whether these differences are accentuated by additional drug treatment remains to be established. Preliminary data (28) suggest that much of the glutathione-*S*-transferase activity occurs in microsomal fractions of WR cells. It is possible that subcellular compartmentalization of enzyme activity may be important in determining drug response.

REFERENCES

1. Goldenberg GJ, and Begleiter A. Alterations of drug transport. *In* Handbook of Experimental Pharmacology: Antitumor Drug Resistance (Fox BW, and Fox M, eds). Heidelberg, West Germany, Springer-Verlag, 1984, pp 198-241.
2. Suzukaka D, Petro BJ, and Vistica DT. Reduction in glutathione content of L-PAM resistant L1210 cell confers drug sensitivity. Biochem Pharmacol 31:121-124, 1982.
3. Calcutt G, and Conners TA. Tumor sulfhydryl levels and sensitivity to the nitrogen mustard merophan. Biochem Pharmacol 12:839-845, 1963.
4. Jakoby WB, and Habig WH. Glutathione transferases. *In* Enzymatic Basis of Detoxification (Jakoby WB, ed). New York, Academic Press, 1984, vol II, pp 63-99.
5. Baars AJ, and Breimer DA. The glutathione-S-transferases, their role in detoxification and toxification of xenobiotics. Ann Biol Chem 38:49-56, 1980.
6. Chasseuad LF. The nature and distribution of enzymes catalyzing the conjugation of glutathione of foreign compounds. Drug Metab Rev 2:185-220, 1973.
7. Tew KD, and Wang AL. Selective cytotoxicity of haloethylnitrosoureas in a carcinoma cell line resistant to bifunctional nitrogen mustards. Molec Pharmacol 21:729-738, 1982.
8. Tew KD, Kyle G, Johnson A, et al. Carbamoylation of glutathione reductase and changes in cellular and chromosome morphology in a cell line resistant to nitrogen mustards but sensitive to nitrosoureas. Cancer Res, 1985. In press.
9. Kaplowitz N, Kuhlenkamp J, and Clifton G. Drug induction of hepatic glutathione-S-transferases in male and female rats. Biochem J 146:351-356, 1975.
10. Guthenberg C, Morgenstern R, DePierre JW, et al. Induction of glutathione-S-transferases A, B, and C in rat liver cytosol by trans-stilbene oxide. Biochem Biophys Acta 631:1-10, 1980.
11. Mannervik B, Guthenberg C, Jenson H, et al. Isozymes of glutathione-S-transferases in rat and human tissues. *In* Functions of Glutathione: Biochemical, Physiological, Toxicological, and Clinical Aspects (Larsson A, Orrenius S, Holmgren A, et al, eds). New York, Raven Press, 1983, pp 75-88.
12. Baars AJ, Jensen M, and Breimer DD. The influence of phenobarbital, 3-methylcholanthrene and 2,3,7,8-tetrachloradibenzo-*p*-dioxin on glutathione-S-transferase activity of rat liver cytosol. Biochem Pharmacol 27:2487-2494, 1978.
13. Tisdale M, and Philips B. Alterations in adenosine 3′,5′-monophosphate-binding protein in Walker carcinoma cells sensitive or resistant to alkylating agents. Biochem Pharmacol 25:1831-1836, 1976.
14. Habig WH, Pabst MJ, and Jakoby WB. Glutathione-S-transferase A: the first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130-7139, 1974.
15. Habig WH, and Jakoby WB. Assays for differentiation of glutathione-S-transferases. *In* Methods in Enzymology. Vol 77. Detoxification and Drug Metabolism: Conjugation and Related Systems (Jakoby WB, ed). New York, Academic Press, 1981, pp 398-405.
16. Benson AM, Talay P, Keen JH, et al. Relationship between soluble glutathione dependent Δ^5-3-ketosteroid isomerase and glutathione-S-transferases of liver. Proc Natl Acad Sci USA 47:158-162, 1977.
17. Griffith OW. Determinations of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem 106:207-212, 1980.
18. Wissinger WL, Estering DN, and Wang RJ. A differential staining technique for simultaneous visualization of mitotic spindle and chromosomes in mammalian cells. Stain Technol 56:221-226, 1981.
19. Mannervik B, and Jenson H. Binary combinations of four protein subunits with different catalytic specifications explain the relationship between six basic glutathione-S-transferases in rat liver cytosol. J Biol Chem 257:9909-9912, 1982.
20. Dent JG, Graichen ME, Roth RW, et al. A radiometric assay for methyl red azo reductase, DT-diaphorase. Anal Biochem 112:299-303, 1981.
21. Beale D, Ketterer B, Carne T, et al. Evidence that Y_a and Y_c subunits of glutathione transferase B (Ligandin) are the products of separate genes. Eur J Biochem 126:459-463, 1982.
22. Black O, and Howerton BK. Glutathione-S-transferase activity in rat pancreas. JNCI 72:121-123, 1984.
23. Lee CG. Biochemical and immunological analysis of an abundant form of glutathione-S-transferase, in mouse testes. Mol Cell Biochem 49:161-168, 1982.
24. Cizosnek H, Sand S, Barker PE, et al. Glutathione-S-transferase Y_a subunit is coded by a multigene family located on a single mouse chromosome. Nucleic Acids Res 12:4825-4833, 1984.
25. Siemann DW. Effect of pretreatment with phenobarbital or SKF 525A on the toxicity and antitumor activity of lomustine. Cancer Treat Rep 67:259-265, 1983.
26. Pickett CB, Telakowski-Hopkins CA, Argenbright L, et al. Regulation of glutathione-S-transferase in RNAs by phenobarbital and 3-methylcholanthrene: analysis using cDNA probes. Biochem Soc Trans 12:71-74, 1984.
27. Tew KD, Moy B, and Hartley-Asp B. Acquired drug resistance is accompanied by modification in karyotype and nuclear matrix of a rat carcinoma cell line. Exp Cell Res 149:443-450, 1983.
28. Monks TJ, Moy BC, and Tew KD. Cytosolic and microsomal glutathione-S-transferase activity in a drug resistant and sensitive cell line. Proc Am Assoc Cancer Res 26:212, 1985.

Stability of the In Vivo P388 Leukemia Model in Evaluation of Antitumor Activity of Natural Products[1]

John C. Marsh,[2,3] Robert H. Shoemaker,[2,*] and Matthew Suffness[2,4]

The stability of the P388 mouse leukemia model over 15 years was examined. Eleven natural products and one synthetic compound (5-FU) tested repetitively under the same conditions of dose and schedule were associated with no significant change in their activity. The P388 model, used in conjunction with other in vivo and in vitro tumor models, continues to be of value in the anticancer drug screening process. [Cancer Treat Rep 69:683–685, 1985]

P388 lymphocytic leukemia in mice (1) has been used by the National Cancer Institute (NCI) since 1968 as the primary screen to detect antitumor activity in crude extracts of plants, microbial fermentation broths, and animal tissues. It has also been used, in conjunction with a cytotoxicity assay, to bioassay fractions to enable isolation of pure materials and also as a preliminary in vivo assay of these isolates. An important characteristic of any such assay, particularly when applied to comparison of data on compounds tested at different times, is its stability.

Because of the large inherent variation in the composition of crude natural product extracts due to differences in time and location of collection, methods of drying and storage, extract preparation, and methods of preparing the material for administration and solubility differences, we decided to evaluate the antitumor response of pure compounds isolated from natural sources as a function of time.

To perform this evaluation, we selected a group of natural products known to have been tested repeatedly over the past 15 years in comparisons with new analogs identified in the NCI drug screening program. In addition, 5-FU was examined, since it has been used as a positive control in the P388 system for many years. Data from > 11,000 assays at a single dose and schedule were available for 5-FU between 1971 and 1983.

MATERIALS AND METHODS

P388 testing performed according to standardized protocols (2,3) and contained in the extensive computerized data base of the NCI drug screening program was reviewed for the years 1967–1984. Test data for 11 natural products and 5-FU were extracted and analyzed. The most frequent testing parameters (drug administration schedule and dose) were determined for each compound (table 1). The minimum number of tests for any one compound was 13 and the median was 26. All agents were administered ip on Day 1 following inoculation of 10^6 tumor cells. All agents were tested in CDF_1 mice except indicine *N*-oxide, which was tested in BDF_1 mice. The percent increase in lifespan (T/C) for these most frequently tested conditions was examined as a function of time. Spearman rank-order correlations of T/C versus time were determined, using the Statistical Analysis System (SAS) on an IBM System 370 computer.

TABLE 1.—Dose and schedule.

NSC No.	Compound	Dose (mg/kg/injection)	Schedule
757	Colchicine	0.25	Daily × 9
3053	Dactinomycin	0.025	Daily × 9
24559	Mithramycin	0.5	Daily × 9
49842	Vinblastine	1.0	Day 1 only
67574	Vincristine	1.0	Day 1 only
123127	Doxorubicin	0.5	Daily × 9
125066	Bleomycin	4.0	Daily × 9
132319	Indicine *N*-oxide	200.0	Daily × 9
141537	Anguidine	1.0	Daily × 9
153858	Maytansine	0.004	Daily × 9
165563	Bruceantin	0.5	Daily × 9
19893	5-FU	20.0	Daily × 9

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[1] Received Oct 23, 1984; accepted Nov 8, 1984.

[2] Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

[3] *Present address:* Departments of Medicine and Pharmacology, Yale University School of Medicine, New Haven, CT.

[4] We thank Dr. Richard M. Simon for helpful statistical advice and Robert H. Brennan for assistance with data management.

[*] *Reprint requests to:* Dr. Robert H. Shoemaker, Drug Evaluation Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Landow Bldg, Rm 5C03, National Institutes of Health, Bethesda, MD 20205.

TABLE 2.—Rank-order correlation of mean T/C and time

Compound	Spearman correlation coefficient	*P* value	No. of observations	T/C*	Years
Colchicine	0.0066	0.966	43	151 ± 22	1973-1984
Dactinomycin	-0.21	0.228	34	178 ± 36	1975-1981
Mithramycin	-0.48	0.098	13	128 ± 21	1968-1980
Vinblastine	-0.13	0.418	39	147 ± 11	1976-1982
Vincristine	-0.04	0.785	48	157 ± 17	1975-1982
Doxorubicin	0.19	0.131	66	182 ± 21	1973-1981
Bleomycin	0.05	0.820	20	113 ± 13	1971-1978
Indicine *N*-oxide	0.31	0.127	26	164 ± 42	1969-1977
Anguidine	0.29	0.172	24	168 ± 24	1971-1981
Maytansine	0.03	0.887	24	143 ± 15	1972-1980
Bruceantin	-0.08	0.761	16	164 ± 16	1973-1981
5-FU	-0.0089	0.334	11,879	182 ± 36	1971-1984

*T/C = mean T/C ± SD. In individual assays, T/C is calculated as the ratio (× 100) of the median survival time of treated animals (T) to the median survival time of control animals (C).

RESULTS

No significant trend over time was observed in the T/C values for any of the natural products examined or for 5-FU (table 2). Correlation coefficients ranged from -0.48 to 0.31, with *P* values ranging from 0.098 to 0.966. Six of the correlation coefficients were negative, while six were positive. The median T/C values for 5-FU for the years 1971-1983 are shown in figure 1. The number of experiments per year ranged from a high of 2624 to a low of three (1974). The small number of experiments conducted in the latter year accounts for the small apparent increase in 5-FU sensitivity for that year shown in figure 1.

DISCUSSION

The results of this study indicate long-term stability of the in vivo P388 mouse leukemia model. Eleven frequently tested natural products as well as one synthetic compound were not associated with any long-term change in the end point (survival) when examined over a total period of 15 years. It is of interest that no upward or downward trend was determined, since the signs of the correlation coefficients were equally divided among the compounds examined. It should be emphasized (*a*) that the drug doses chosen for our evaluation were those most frequently used, but were not necessarily the optimal doses as determined from formal dose-response studies, and (*b*) that there may be substantial variation in drug efficacy in the P388 model at any given time. The value of stability in drug screening models has long been recognized and specific procedures have been developed to maximize this feature of the NCI tumor models. One of the most important procedures is the periodic replacement of the cell stock utilized in the screening laboratories. By maintaining a central tumor repository with a frozen bank of cells which are thawed, implanted in mice, and shipped to

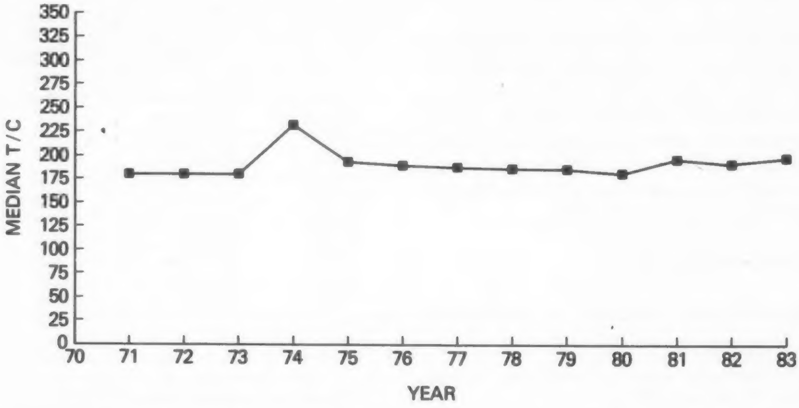

FIGURE 1.—Plot of median T/C by year for 5-FU. Total of 11,878 individual P388 assays were conducted between 1971 and 1983. Median T/C values are plotted for these years.

screening laboratories at regular intervals, the need for protracted serial passage in any one laboratory and the consequent risk of genetic drift has been eliminated.

While it is reassuring that this in vivo model, as well as the in vitro KB cell line (4), has been stable in their response to known active antitumor agents over a long period of time, it is probable that the screening process to detect anticancer drugs for ultimate clinical use can be substantially improved. To that end, new models such as the in vitro human tumor colony-forming assay are being developed and evaluated by the NCI. Several compounds that do not have activity in the P388 system have already been identified with this latter model (5). Human tumor cell lines may also be of use in this regard and, if so, long-term stability will remain an important characteristic.

REFERENCES

1. DAWE CJ, and POTTER M. Morphologic and biologic progression of a lymphoid neoplasm of the mouse in vivo and in vitro. Am J Pathol 33:603, 1957.
2. GERAN RI, GREENBERG NH, MACDONALD MM, ET AL. Protocols for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemother Rep Part 3, vol 3(2):1-103, 1972.
3. In Vivo Cancer Models. NIH Publication No. 84-2635. US Department of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda, MD, 1984.
4. SHOEMAKER RH, ABBOTT BJ, MACDONALD MM, ET AL. Use of the KB cell line for in vitro cytotoxicity assays. Cancer Treat Rep 67:97, 1983.
5. SHOEMAKER RH, WOLPERT-DEFILIPPES MK, MELNICK NR, ET AL. Recent results of new drug screening trials with a human tumor forming assay. *In* Human Tumor Cloning (Salmon S, and Trent J, eds). Orlando, Grune and Stratton, 1984, pp 345-355.

Leukemic Meningitis in B-Cell Chronic Lymphocytic Leukemia[1]

James P. Steinberg, Michael Pecora, and J. L. Lokey[2,3,*]

Symptomatic meningeal involvement is rare in chronic lymphocytic leukemia (CLL). Hansen (1) reviewed 189 consecutive cases of CLL and found no definite examples of leukemic meningitis. Getaz and Miller (2) reported the only case of symptomatic CNS involvement in a patient with B-cell CLL, the cell line from which > 90% of cases of CLL arise. Their patient had perivascular spinal cord infiltration with an isolated focus of meningeal involvement and died before the effects of therapy could be observed. In the rare T-cell CLL variant, six of 52 patients had leukemic infiltration of the meninges (3,4). In two cases of meningeal leukemia complicating CLL, lymphocyte surface markers were not studied (5). We will describe a patient with B-cell CLL who had leukemic meningitis and showed a favorable response to intrathecal methotrexate and whole-brain irradiation.

CASE REPORT

A 38-year-old male presented with anorexia, progressive dyspnea on exertion, and a 20-pound weight loss over 6 months. Diffuse lymphadenopathy and marked hepatomegaly were present. The wbc count was 71,000/mm^3 (with 93% mature lymphocytes), hemoglobin was 11.2 g/dl, hematocrit was 34%, and platelet count was 259,000/mm^3. Anticomplement Coombs' test was positive. Bone marrow was hypercellular with 70%-75% mature lymphocytes, some with cleaved nuclei. Ninety percent of the lymphocytes were B cells, which stained positively with kappa antisera. The intensity of the immunoperoxidase staining was relatively high, consistent with CLL. B-Cell CLL was diagnosed; biweekly chlorambucil and prednisone were begun, producing a rapid decrease in wbc count. Seven weeks later the patient was readmitted with fatigue and progressive anemia. He was mildly lethargic and had a temperature of 100.5°F. The neck was supple. There was bilateral proptosis and periorbital puffiness. The pupils, extraoccular movements, and fundi were normal. Lymphadenopathy and splenomegaly had diminished. Laboratory studies showed a wbc count of 5000/mm^3, with 57% lymphocytes, 37% segmented neutrophils, and 2% monocytes; hemoglobin of 6.6 g/dl; hematocrit of 18.7%; and platelet count of 298,000/mm^3. Thyroid function tests were normal; antithyroglobulin and antimicrosomal antibodies were not detected. The CSF protein was 107 mg/dl, glucose was 46 mg/dl, and wbc count was 170/mm^3 (100% lymphocytes with 4 rbcs/mm^3). CSF stains and cultures for bacteria, fungus, and mycrobacteria were negative. Cryptococcal antigen was not detected in the CSF from any of three lumbar punctures. There was no evidence of infection on frequency-pulsed, electron-capture, gas-liquid chromatography (6). Lymphocyte typing of the CSF revealed many B lymphocytes, almost all staining with kappa antisera, again with relatively high intensity. Computed cranial tomography showed cloudy infiltration behind both eyes and in each maxillary sinus. A maxillary sinus biopsy was performed and showed a moderate lymphocytic infiltrate composed of mature lymphoid cells.

Whole-brain irradiation and intrathecal methotrexate were instituted. After 1 month of treatment, his lethargy and apathy resolved. A lumbar puncture showed no pleocytosis, with a protein of 34 mg/dl and a glucose of 51 mg/dl. The retro-orbital infiltrates were absent on a follow-up computerized tomographic scan. The patient was maintained on monthly intrathecal methotrexate treatments, with no evidence of CNS relapse. Diffuse lymphadenopathy and weight loss persisted. Five months later systemic chemotherapy was changed to cyclophosphamide, vincristine, prednisone, and procarbazine, with some initial clinical improvement.

Several months later, approximately 1 year after diagnosis, the patient developed gram-negative septic shock and died. Wbc count was 10,300/mm^3, with 95% lymphocytes. Post-mortem examination showed no meningeal leukemia. An extensive infiltrate of small lymphocytes was noted in bone marrow. The lymph nodes at post-mor-

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[2] Crawford W. Long Memorial Hospital, and Department of Internal Medicine, Division of Oncology/Hematology, Emory University School of Medicine, Atlanta, GA.

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[*] *Reprint requests to:* Dr. J. L. Lokey, Georgia Oncology Hematology Clinic, PC, 25 Prescott St, Suite 6401, Atlanta, GA 30308.

tem examination contained diffuse atypical lymphocytic infiltrates and loss of normal architecture.

DISCUSSION

The patient's age (38 years) and the aggressive nature of his disease are atypical for CLL. Morphologically, most lymphoid cells in both peripheral blood and bone marrow were small round cells with a high nuclear-cytoplasmic ratio and densely clumped nuclear chromatin typical of CLL. However, a subpopulation of cells with prominent nuclear folds and clefts was noted.

A question raised by this case is why does B-cell CLL, unlike T-cell CLL, produce symptomatic meningeal involvement so infrequently? Manconi et al (7) and later Gangzi et al (8) demonstrated that most lymphocytes in normal CSF are T lymphocytes. Both investigators attributed their findings to the greater recirculating capacity of T lymphocytes. This migratory ability has been mentioned in conjunction with the T-cell CLL's predilection for the CNS (3).

Migration of circulating leukemic cells through venous endothelium was suggested by Price and Johnson (9) as a mechanism of meningeal spread in acute lymphocytic leukemia. They reviewed 126 autopsies of children with acute lymphocytic leukemia and found that leukemic cells first appeared in the walls of the superficial arachnoid veins and that more extensive leukemic infiltration did not occur in the absence of this perivascular involvement.

A necropsy series found leukemic infiltration of the CNS in ten of 14 cases of CLL (10). Unfortunately, the extent of the CNS infiltrates was not clearly reported. None of these patients had symptomatic CNS disease requiring treatment. Unless most of these patients had the uncommon T-cell CLL, asymptomatic leukemic infiltration of the CNS in B-cell CLL is common, at least preterminally. This series suggests that the higher incidence of symptomatic T-cell CLL leukemic meningitis may not be related to greater T-cell CLL migration into the CNS but to more aggressive behavior after CNS infiltration. In CLL, leukemic infiltration into the periorbital areas is uncommon (1). Paranasal sinus carcinoma can infiltrate the leptomeninges by direct extension (11). In our patient, contiguous spread to the leptomeninges from the retro-orbital area or the paranasal sinuses is also possible.

In conclusion, meningeal leukemia can complicate B-cell CLL and can successfully be treated with intrathecal methotrexate and whole-brain irradiation. The difference between the high prevalence of CNS involvement at autopsy and the low incidence of symptomatic CNS disease in B-cell CLL is interesting and suggests that after meningeal infiltration, B-cell CLL is usually indolent. However, since CNS involvement is not commonly associated with CLL, this difference may in part reflect a failure of clinicians to consider the diagnosis of leukemic meningitis when patients with CLL present with subtle neurologic abnormalities.

REFERENCES

1. HANSEN NM. Chronic lymphocytic leukemia. Scand J. Haematol 18:9-283, 1973.
2. GETAZ EP, and MILLER GJ. Spinal cord involvement in chronic lymphocytic leukemia. Cancer 43:1858-1861, 1979.
3. DAVIES GE. Letter to the editor. Cancer 50:605-606, 1982.
4. REINHERZ EL, NADLER LM, ROSENTHAL DS, ET AL. T-Cell subset characterization of human T-CLL. Blood 53:1066-1075, 1979.
5. LIEPMAN MK, and VOTAW ML. Meningeal leukemia complicating chronic lymphocytic leukemia. Cancer 47:2482-2484, 1981.
6. EDMAN DC, CRAVEN RB, and BROOKS JB. Gas chromatography in the identification of micro-organisms and diagnosis of infectious diseases. CRC Crit Rev Clin Lab Sci 14:133, 1981.
7. MANCONI PE, ZACCHEO D, BUGIANI O, ET AL. T and B lymphocytes in normal cerebrospinal fluid. N Engl J Med 249:49, 1976.
8. GANGZI D, COLLARD-RONGE E, BALLERIAUX-WAHA D, ET AL. T lymphocytes in cerebrospinal fluid. N Engl J Med 296:902, 1976.
9. PRICE RA, and JOHNSON WW. The central nervous system in childhood leukemia. I. The arachnoid. Cancer 31:520-533, 1973.
10. RESKE-NEILSEN E, PETERSEN JH, SOGUARD H, ET AL. Leukemia of the central nervous system. Lancet 1:211-212, 1974.
11. KOKKORIS CP. Leptomeningeal carcinomatosis. How does cancer reach the pia-arachnoid? Cancer 51:154-160, 1983.

Six-Drug Chemotherapy (Hexamethylmelamine, Doxorubicin, Cisplatin, Cyclophosphamide, Methotrexate, and 5-FU; CHAMP-5) for Ovarian Carcinoma: Alternating Sequences of Combination Regimens[1,2]

Mark W. Pasmantier, Morton Coleman,* Richard T. Silver, and W. Perry Ballard[3]

Twenty-five patients with previously untreated adenocarcinoma of the ovary (International Federation of Gynecology and Obstetrics, stages II, III, and IV) were placed on a six-drug trial (CHAMP-5) for 18 months. This trial consisted of 28-day cycles of hexamethylmelamine, doxorubicin, and cisplatin alternating with hexamethylmelamine, cyclophosphamide, methotrexate, and 5-FU. After a minimum follow-up of 31 months, 14 (56%) of the 25 evaluable patients achieved complete or partial response and nine (36%) achieved complete pathologic response. Although CHAMP-5 is effective in the treatment of ovarian cancer, the results in these patients are not substantially different from those achieved with hexamethylmelamine, doxorubicin, and cisplatin alone. [Cancer Treat Rep 69:689–693, 1985]

Ovarian cancer is the fifth most frequent cause of cancer death in women. Five-year survival rates are between 30% and 35%, since approximately 80% of patients have advanced stage III and IV disease at the time of diagnosis (1,2). For these patients with advanced disease, chemotherapy remains the mainstay of treatment (3).

A number of drugs have proven efficacy in ovarian cancer either alone or in combination. Six agents reported to be effective in ovarian cancer include cyclophosphamide, hexamethylmelamine, doxorubicin, methotrexate, cisplatin, and 5-FU (CHAMP-5) (3-6). Hexamethylmelamine, doxorubicin, and cisplatin (HAC) in combination has been shown to be an effective regimen for advanced ovarian carcinoma (7-9). Response frequencies of 50%-70% in previously treated and untreated patients, respectively, have been achieved. A combination of hexamethylmelamine, cyclophosphamide, methotrexate, and 5-FU (Hexa-CAF) has achieved a somewhat similar response in 40 women with untreated adenocarcinoma of the ovary (10). The median survival was 29 months with Hexa-CAF.

The CHAMP-5 regimen consists of alternating sequences of these two combinations (HAC and Hexa-CAF) in ovarian carcinoma. The two combinations, although sharing the agent hexamethylmelamine, have otherwise different agents with differing mechanisms of action. Reported herein are the results of the CHAMP-5 protocol in 25 women with previously untreated carcinoma of the ovary, with a minimum follow-up of 31 months.

PATIENTS AND METHODS

Patient Selection

From January 1979 to October 1980, 25 patients with adenocarcinoma of the ovary at The New York Hospital-Cornell Medical Center were studied on the CHAMP-5 protocol. Patient eligibility, debulking procedures, and procurement of informed consent were as previously described (10).

Treatment

HAC Regimen

Drug cycles were repeated every 56 days for a total of nine cycles, beginning with doxorubicin and cisplatin. During the 4 hours preceding treatment, patients were given 1-2 L of iv fluid (usually 5% dextrose in 0.45% saline). When the urine output increased to > 100 ml/hour,

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[3] Oncology Service, Division of Hematology-Oncology, Department of Medicine, The New York Hospital-Cornell University Medical Center, New York, NY.

**Reprint requests to:* Morton Coleman, MD, Oncology Service, Division of Hematology-Oncology, Department of Medicine, The New York Hospital-Cornell Medical Center, 525 E 68th St, New York, NY 10021.

doxorubicin (30 mg/m^2) was given iv over 30 minutes. This was followed by iv furosemide (20 mg). Cisplatin (60 mg/m^2) was then infused over 1 hour. Urine output was maintained at 100 ml/hour for at least 4 hours after the cisplatin was given, using iv furosemide when necessary.

Hexamethylmelamine (200 mg/m^2) given orally was administered on Days 7-21 of each cycle. Most of the dose was given at bedtime whenever possible. Doses of doxorubicin and hexamethylmelamine were modified for myelosuppression and hepatotoxicity as previously described (8).

The values of BUN, creatinine, and creatinine clearance were measured prior to the administration of cisplatin. Those patients with a creatinine clearance $< 50\%$ of predicted or a creatinine > 1.5 mg/dl received doxorubicin and hexamethylmelamine, but did not receive additional cisplatin until renal function improved to acceptable values. In some patients, improvement of renal function occurred with hydration only.

The maximum cumulative dose of doxorubicin given was 550 mg/m^2. In any patient with signs of cardiotoxicity, doxorubicin was discontinued, but cisplatin and hexamethylmelamine were continued.

Patients with moderate to severe hexamethylmelamine-induced vomiting were given 50% of the calculated dose. Hexamethylmelamine was discontinued in any patient developing gastrointestinal hemorrhage or intractable vomiting related to the drug. Patients with paresthesia of moderate severity were given 50% of the calculated dose of hexamethylmelamine. Hexamethylmelamine was discontinued in any patient with severe degrees of paresthesia, weakness, hallucinations, Parkinsonian symptoms, or petit mal seizures.

Hexa-CAF Regimen

The Hexa-CAF portion of the regimen was administered every alternate 56 days, 28 days following each HAC cycle. Each of the nine cycles was instituted on Day 1 with an iv push of 5-FU (600 mg/m^2) and methotrexate (40 mg/m^2). The iv medications were repeated on Day 8. Cyclophosphamide and hexamethylmelamine were given orally at doses of 150 mg/m^2 each on Days 1-14 of the cycle. The dose modifications for Hexa-CAF were as previously described (8).

Response Evaluation

Criteria for progressive disease, no change, and partial response (PR; a 50% reduction in tumor) have been previously described (8). Complete clinical response (CCR) consisted of the clinical disappearance of all physical, sonographic, and computerized tomographic evidence of tumor. In patients completing 18 cycles of chemotherapy who were in CCR, a "second-look" laparotomy, as has been described, was required to document the absence of disease, a complete pathologic response (CPR) (8). Chemotherapy was then discontinued.

Toxicity Evaluation

All patients were evaluated systematically for toxicity. The major toxic effects were gastrointestinal, hematopoietic, neurologic, and renal. Toxicity was graded from 0 (no toxicity) to 4 (severe, life-threatening), as has been described (8).

RESULTS

Response

Of the 25 patients, 14 had response (56%) and 11 had progressive disease (table 1). Of the 14 responses, two were partial and 12 were CCRs. Of the 12 patients who had CCR, 11 underwent surgical re-evaluation (one died of a myocardial infarction prior to exploration); seven

TABLE 1.—Response by variables*

	Total	Response†					Response rate (%)	Median survival (mos)
		CCR	CPR	PR	NC	PD		
Total	25	12	7	2	0	11	56	18
Age (yrs)								
< 60	14	8	5	0	0	6	57	22
> 60	11	4	2	2	0	5	55	12
Stage								
II	4	2	1	1	0	1	75	12
III	11	5	3	1	0	5	55	30
IV	10	5	3	0	0	5	50	18
Ascites								
Yes	15	6	3	1	0	8	47	18
No	10	6	4	1	0	3	70	25

* Values = No. of patients.

†NC = no change; and PD = progressive disease.

had CPR; and four had persistent disease. Of the four women with persistent disease, two are now free of disease after the administration of additional chemotherapy. Both of these patients had negative second, second-look ("third-look") laparotomies. The other two patients died with advanced disease.

Survival

The median survival was 18 months for the entire group, 31 months for responders, and 12 months for nonresponders.

Response by Variables

Age.—The difference in response frequency between patients < 60 years of age and patients > 60 years of age was not significant. Of the 14 women < 60 years of age, there were eight responders (57%), five achieving CPR (table 1). Of the 11 women > 60 years of age, there were six responders (55%); however, only two older women achieved CPR.

Ascites.—Of the 25 patients, 15 had ascites (table 1); of these 15, seven (47%) had a response to CHAMP-5 therapy, whereas seven of ten (70%) without ascites responded.

Stage.—Of the 25 patients in this study, four had stage II, 11 had stage III, and ten had stage IV disease (table 1). Of the 21 patients with advanced stages of disease (stages III and IV), one achieved PR and ten achieved CCR; six of the ten achieved CPR. All six patients with CPR remain free of disease. Of the four women who achieved CCR, one is now free of disease because of additional therapy, while the other three relapsed and died. Of the four women with stage II ovarian carcinoma, two achieved CCR and one achieved PR. Both patients with CCR underwent a second-look laparotomy; one proved negative, while the other had positive cytology. This woman had additional treatment; she was found clinically free of disease and had another laparotomy performed at which time she achieved CPR. Both patients who achieved CPR remain free of disease. The patient who achieved PR and a woman with progressive disease died shortly afterwards of advanced ovarian cancer.

Toxicity

Toxicity was predominantly gastrointestinal and hematologic (table 2).

Gastrointestinal.—All 25 patients had vomiting on the day of cisplatin administration (grade 2). Nausea was only minimally relieved with antiemetics. In addition, all patients who received hexamethylmelamine experienced mild to moderate nausea.

Myelosuppression.—Fourteen patients experienced moderate to life-threatening myelosuppression at some time during the treatment. One woman died from overwhelming infection as a result of severe leukopenia (grade 4). Nine patients had thrombocytopenia. No patient hemorrhaged as a result of the lowered platelet count.

Neurotoxicity.—Four patients had neurotoxic complications (table 2), presumably due to either the hexamethylmelamine, the cisplatin, or both, but only one woman had severe complications (grade 3). This consisted of marked paresthesia and loss of position sense; she was unable to write or to feed herself. The other three women had mild to moderate peripheral neuropathy (grade 2) and only mild muscular weakness.

Nephrotoxicity.—Six patients experienced some significant degree of nephrotoxicity (table 2). The creatinine clearance of one patient fell to < 25% of her normal level (grade 3 toxicity), causing interruption of treatment until the creatinine clearance returned to normal. No chemotherapy treatment was delayed by > 1 month because of renal toxicity.

Other toxic effects.—One woman experienced a grade 3 hearing loss due to administration of cisplatin. During the course of her therapy the range of her hearing fluctuated from normal to a severe toxic level (grade 3).

DISCUSSION

The combination chemotherapy regimens of HAC and

TABLE 2.—Toxicity*

Toxicity	Total	Grade 1	2	3	4
Gastrointestinal	25	0	25	0	0
Myelosuppression					
Leukopenia	14	0	6	6	2
Thrombocytopenia	9	2	3	1	3
Neurotoxicity	4	1	2	1	0
Nephrotoxicity	6	4	1	1	0
Cystitis	6	4	1	1	0

* Values = No. of patients.

Hexa-CAF, when used separately in treating adenocarcinoma of the ovary, have both produced high response frequencies. Response frequencies approximating 50%-70% or even better have been reported by Brower et al (7,8) and Vogl et al (9) using HAC and by Young et al (10) using Hexa-CAF. Because of these responses, it seemed logical to test these two combinations in alternating fashion in our CHAMP-5 program. Our enthusiasm for using alternating regimens was augmented by our similar studies in Hodgkin's disease and the later publication of the Goldie-Coldman hypothesis (11-14). This hypothetical model suggests that using alternating regimens may be valuable for treating the spontaneous emergence of resistant cells, thereby enhancing the possibility of eliminating the last viable cancer cell. Partial clinical testing of this hypothesis has already been published in Hodgkin's disease (13-16). In contrast to some preliminary favorable reports in Hodgkin's disease, the overall response frequency here was a disappointing 56% (15,16). The response was not only considerably less than anticipated but was, in fact, somewhat less than that achieved with HAC alone in untreated patients.

The disparity between our results now using alternating regimens in ovarian cancer and those initially reported may have several explanations (17). Hexa-CAF may not be truly non-cross-resistant with HAC or may not be as effective as HAC. The employment of one less effective combination in alternating fashion with a more effective regimen may simply be less effective than using the better regimen all that time (9). Second, the two tumors (ovarian cancer and Hodgkin's disease) may both not be equally good models for testing the effectiveness of the Goldie-Coldman hypothesis. It would appear, nevertheless, that ovarian cancer may be as good or an even better testing model than Hodgkin's disease since ovarian tumor, while being responsive to chemotherapy, is less so than Hodgkin's disease, thus requiring a considerably shorter time to determine true effectiveness of various regimens. Results of a randomized controlled long-term study in Hodgkin's disease have also shown no statistical differences between the use of single regimens and two regimens in alternating sequence (13,14).

There still may be merit to using alternating sequences of chemotherapy regimens, if only to reduce toxicity to any one organ system, provided the combination regimens are directed primarily at different organ systems. In Hodgkin's disease this has occurred, enhancing the ability to administer more overall chemotherapy (13). It did not, however, result clinically in the theoretical advantage of enhanced response (13,14). The overall toxicity of the CHAMP-5 protocol not unexpectedly fell between that found in the HAC and the Hexa-CAF protocols. Twenty-eight percent of the patients receiving Hexa-CAF experienced severe toxicity (grades 3 and 4), whereas only 10% of patients receiving HAC had severe toxicity (7,8,10). The CHAMP-5 regimen produced a frequency of severe toxicity in 15% of patients. The somewhat higher toxicity was primarily hematopoietic, a toxic effect which is shared by both regimens. On the other hand, only one treatment delay because of grade 3 nephrotoxicity occurred in this study, reflecting the fact that with alternating sequences, platinum is given on alternate months.

Our results continue to emphasize the importance of second-look laparotomies. Of 11 patients achieving CCR who had laparotomy, only seven were found to have CPR. Two of the four patients who were found to have persistent disease at initial second-look laparotomy became free of disease after additional chemotherapy for 12 months. Subsequently, these two patients had negative second, second-look (third-look) laparotomies.

Despite our disclaimers, a CPR rate of 28% still remains an encouraging achievement, since CPR offers the potential of cure. Furthermore, if the two patients who were found to be free of disease at second, second-look laparotomy are included, the CPR frequency totals 36%, identical to that initially achieved with Hexa-CAF, but with considerably reduced toxicity. Hopefully, further refinements in our chemotherapy techniques, employing alternate or additional hypothetical models, will aid us in the pursuit of cure for this important malignancy (18-21).

REFERENCES

1. Cancer Facts and Figures 1980. American Cancer Society Publication, New York, 1980.
2. CUTLER SJ, MYERS MH, and GREEN SB. Trends in survival rates of patients with cancer. N Engl J Med 293:122-124, 1975.
3. YOUNG RC. Chemotherapy of ovarian cancer: past and present. Semin Oncol 2:267-276, 1975.
4. WAMPLER GL, MELLETTE SJ, KUPERMINC M, ET AL. Hexamethylmelamine (NSC-13875) in the treatment of advanced cancer. Cancer Chemother Rep 56:505-514, 1972.
5. DE PALO GM, DE LENA M, DI RE F, ET AL. Melphalan versus adriamycin in the treatment of advanced carcinoma of the ovary. Surg Gynecol Obstet 141:899-902, 1975.
6. GOTTLIEB JA, and DREWINKO B. Review of the current clinical status of platinum coordination complexes in cancer chemotherapy. Cancer Chemother Rep 59:621-628, 1975.
7. BROWER MS, COLEMAN M, CHIARIERI DR, ET AL. Combination chemotherapy of ovarian carcinoma. Proc Am Assoc Cancer Res and ASCO 20:751, 1979.
8. BROWER MS, COLEMAN M, PASMANTIER MW, ET AL. Treatment of advanced ovarian carcinoma with hexamethylmelamine, doxorubicin, and *cis*-platinum (HAC): results in both untreated and previously treated patients. Med Pediatr Oncol 12:17-24, 1984.
9. VOGL SE, PAGANO M, KAPLAN BH, ET AL. Combination chemotherapy of advanced ovarian cancer with hexamethylmelamine, cisplatinum and doxorubicin after failure of prior therapy. Obstet Gynecol 56:635-640, 1980.
10. YOUNG RC, CHABNER BA, HUBBARD SP, ET AL. Advanced ovarian adenocarcinoma. A prospective clinical trial of melphalan (L-PAM) versus combination chemotherapy. N Engl J Med 299:1261-1266, 1978.
11. GOLDIE JH, and COLDMAN AJ. A mathematic model for relating the drug sensitivity of tumors to their spontaneous mutation rate. Cancer Treat Rep 63:1727-1733, 1979.

12. GOLDIE JH, COLDMAN AJ, and GUDAUSKAS GA. Rationale for the use of alternating non-cross-resistant chemotherapy. Cancer Treat Rep 66:439-449, 1982.
13. VINCIGUERRA VP, COLEMAN M, and RAFLA S. A randomized trial of chemoimmunotherapy in advanced, recurrent Hodgkin's disease. Proc Am Assoc Cancer Res and ASCO 19:C-57, 1978.
14. BLOOMFIELD CD, PAJAK TF, GLICKSMAN AS, ET AL. Chemotherapy and combined modality therapy for Hodgkin's disease: a progress report on Cancer and Leukemia Group B studies. Cancer Treat Rep 66:835-846, 1982.
15. BONADONNA G, SANTORO A, BONFANTE V, ET AL. Cyclic delivery of MOPP and ABVD combinations in stage IV Hodgkin's disease: rationale, background studies, and recent results. Cancer Treat Rep 66:881-887, 1982.
16. SANTORO A, BONADONNA G, BONFANTE V, ET AL. Alternating drug combinations in the treatment of advanced Hodgkin's disease. N Engl J Med 306:770-775, 1982.
17. BROWER MS, PASMANTIER MW, COLEMAN M, EL AL. Six drug CHAMP-5 combination chemotherapy of ovarian carcinoma. Proc Am Assoc Cancer Res and ASCO 21:C-191, 1980.
18. NORTON L, and SIMON R. Tumor size, sensitivity to therapy, and design of treatment schedules. Cancer Treat Rep 61:1307-1317, 1977.
19. NORTON L, and SIMON R. New thoughts on the relationship of tumor growth characteristics to sensitivity to treatment. *In* Methods in Cancer Research XVII: Cancer Drug Development Part B (DeVita VT, and Busch H, eds). New York, Academic Press, 1979, pp 53-90.
20. PASMANTIER MW, COLEMAN M, BALLARD WP, ET AL. HAC-cyclophosphamide treatment of ovarian carcinoma: alternating intensification chemotherapy. Proc ASCO 3:C-675, 1984.
21. COLEMAN M, PASMANTIER MW, and SILVER RT. HAC-Cytoxan chemotherapy for ovarian carcinoma: alternating chemotherapy with intensification. Cancer 55:40-45, 1985.

Cisplatin-Containing Chemotherapy in the Treatment of Invasive Thymoma: Report of Five Cases[1]

Giuseppe Giaccone,* Roberto Musella, Oscar Bertetto, Michela Donadio, and Alessandro Calciati[2]

Invasive thymomas are rare neoplasms arising from the epithelial cells of the thymus. In this paper, we report five cases of invasive thymoma treated with cisplatin either alone or combined with etoposide. Two partial responses (lasting 1 and 27+ months from the start of chemotherapy), two minor responses (lasting 1.5 and 13+ months from the start of chemotherapy), and one mixed response were observed. Further studies with cisplatin-containing regimens are warranted in the treatment of this rare tumor. [Cancer Treat Rep 69:695–697, 1985]

Thymomas represent about 17% of mediastinal enlargements and are defined as neoplasms which arise from the epithelial cells of the thymus gland. Thirty percent of thymomas are considered malignant on the basis of macroscopic invasiveness; most malignant thymomas are characterized by local tissue invasion or local recurrence, but 1% will develop extrathoracic metastases.

Surgery alone is usually curative in a completely encapsulated neoplasm; radiotherapy is reserved for patients with residual disease after surgery or with invasive thymomas. The role of chemotherapy in invasive thymomas is not yet well defined, partially due to the rarity of this malignancy. There are limited reports describing objective responses to the use of cytotoxic drugs. Among these drugs, cisplatin (DDP), either alone or in combination regimens, has achieved some long-lasting partial and complete responses in five patients with invasive thymoma (1–5).

This paper describes our experience of treatment of invasive thymomas with DDP in five additional patients. Since the combination of DDP and etoposide has been reported to be superior to either drug alone, both in animal tumor systems and for several human neoplasms, four of our patients have been treated with DDP plus etoposide, although no data are available on the use of etoposide alone in invasive thymoma.

PATIENTS AND METHODS

Five patients with invasive thymoma, initially seen between November 1980 and September 1983, were treated with these regimens at our institution. Patient characteristics, prior treatment status, and chemotherapy outcome are described in table 1. Two patients (Nos. 1 and 2) had relapsed after a radical excision and following a macroscopically incomplete resection. Three patients had bulky disease at surgery; no patient presented with myasthenia gravis. Symptoms at diagnosis were dyspnea (two patients), chest pain (two), and superior vena cava syndrome, dyspnea, and hoarseness (two).

The only patient who had previously received chemotherapy before being treated with DDP and etoposide (No. 1) had experienced a partial response with bleomycin, cyclophosphamide, doxorubicin, vincristine, and prednisone in extrathoracic disease. He then received a combination of cyclophosphamide, vincristine, and prednisone, with no change in his tumor, immediately prior to treatment with DDP and etoposide. Cisplatin was administered at 60 mg/m^2 iv on Day 1, with etoposide given at 120 mg/m^2 on Days 4, 6, and 8 in Patients 1–4. Based on a recent report describing a long-lasting complete response to high-dose DDP (HD-DDP) alone (5), Patient 5 received DDP as a single agent at doses of 100–130 mg/m^2. DDP was administered along with a program of hydration with 2 L of iv fluids and 250 ml of a 20% mannitol solution; DDP was diluted in 250 ml of normal saline and the hydration lasted 4–5 hours. The patient treated with HD-DDP received 2 L of normal saline prehydration starting the night before chemotherapy. Cycles were repeated every 4 weeks. Responses were de-

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[2] Ospedale S. Giovanni, Antica Sede, Divisione di Oncologia Medica, Torino, Italy.

**Reprint requests to:* Dr. Giuseppe Giaccone, Divisione di Oncologia Medica, Ospedale S. Giovanni, Antica Sede, V. Cavour 31, 10123 Torino, Italy.

TABLE 1.—Patient characteristics, prior treatment, and outcome of chemotherapy

Characteristics	Patient No.— 1	2	3	4	5
Age at diagnosis (yrs)	54	46	66	72	52
Sex	Male	Female	Male	Male	Male
Performance status[*]	1	2	3	2	2
Histology[†]	LE	E	E	E	LE
Extrathoracic disease	Yes	Yes	No	No	No
Prior surgery	Radical	Incomplete	Palliative	Biopsy	Biopsy
Prior radiotherapy	Yes	Yes	No	No	No
Prior chemotherapy	Yes	No	No	No	No
Chemotherapy	DDP + etoposide	DDP + etoposide	DDP + etoposide	DDP + etoposide	High-dose DDP
No. of cycles	4	4	3	5	3
Response[‡]	MX	PR	MR	MR	PR
Response duration (mos)	2	27+	1.5	13+	1

[*] Eastern Cooperative Oncology Group criteria.

[†] LE = lymphoepithelial type; and E = predominantly epithelial type.

[‡] MX = mixed response; PR = partial response; and MR = minor response.

fined as follows: *complete response*—complete disappearance of tumor for at least 4 weeks; *partial response*—reduction of > 50% of tumor mass for at least 4 weeks; *minor response*—reduction of < 50% but > 25% of tumor for at least 4 weeks; *no change*—a < 25% increase or reduction of tumor mass; and *disease progression*—increase of > 25% of tumor mass.

RESULTS

Some evidence of antitumor activity could be seen in all patients. The patient treated with HD-DDP (No. 5) attained a partial response in the mediastinal tumor after the first 100 mg/m^2 of DDP, but the response lasted only 1 month. Four patients (Nos. 1-4) were given the DDP plus etoposide regimen. Patient 2 obtained a partial response of multiple pleural and pulmonary metastases; the regression of tumor masses continues 23 months after suspension of chemotherapy following four courses of treatment, despite the patient's own decision to discontinue chemotherapy. A breast nodule, present at first examination, was excised 10 months after the start of chemotherapy, and the histology was in accordance with the diagnosis of thymoma. At present, 27 months after initiation of chemotherapy, only a few pleural plaques can be detected by chest computerized tomography. Patient 1 had complete disappearance of axillary and supraclavicular adenopathy for 2 months, but no change in bulky abdominal disease (mixed response). Patients 3 and 4 had minor responses, one involving mediastinal tumor and the second involving multiple pulmonary metastases; this second patient eventually developed cavitation of one pulmonary lesion and died of septic complications unrelated to chemotherapy-induced leukopenia. Chemotherapy was discontinued in Patient 4 after five courses due to poor gastrointestinal tolerance.

The toxicity encountered consisted of nausea and vomiting in all patients, a temporary elevation of serum creatinine level in two (maximum 1.7 mg/dl for 1 week), and persistent ototoxicity in the patient treated with HD-DDP alone. Marrow toxicity was primarily leukopenia that was never severe, but was long-lasting in two patients (Nos. 1 and 2) who had been previously irradiated; this toxicity caused a slight delay of chemotherapy. Alopecia developed in all patients given DDP with etoposide, and diarrhea occurred in two instances.

DISCUSSION

Surgery and radiotherapy have been adequately evaluated in the management of malignant thymomas, but only a few reports deal with chemotherapy. Remissions of short duration have been reported with cyclophosphamide, doxorubicin, and maytansine; the longest response to a single agent has been described with cisplatin (1-3,5). Several regimens have been reported to be active, either with or without DDP, although the total number of patients reported with DDP is only five. Although etoposide has not been previously tested as a single agent in malignant thymoma, there is evidence that the combination of this agent with DDP is more effective than either drug alone in several human tumors. For this reason, the combination was tested in four of our five patients. In all five patients reported in this trial, some degree of tumor shrinkage could be demonstrated. In one patient, who attained a partial response after four courses of DDP plus etoposide, the tumor regression continued after suspension of chemotherapy and only a small amount of tumor remains.

Our experience adds further evidence to the activity of DDP either alone or in combination in patients with malignant thymoma. Although DDP and DDP-containing combinations seem to be useful in treating patients with this rare disease, further study is still needed and the role of etoposide is still to be determined.

REFERENCES

1. TALLEY RW, O'BRIAN RM, GUTTERMAN JU, ET AL. Clinical evaluation of toxic effects of *cis*-diammine-dichloroplatinum (NSC-119875). Phase I clinical study. Cancer Chemother Rep 57:465–471, 1973.
2. NEEDLES B, KEMENY N, and URMACHER C. Malignant thymoma with renal metastases responding to cisplatin. Cancer 48:223–226, 1981.
3. SHETTY MR, and ARORA R. Invasive thymoma treated with cisplatin. Cancer Treat Rep 65:531, 1981.
4. CAMPBELL MG, POLLAND R, and AL-SARRAF M. A complete response in metastatic malignant thymoma to cisplatin, doxorubicin, and cyclophosphamide. Cancer 48:1315–1317, 1981.
5. COCCONI G, BONI C, and CUOMO A. Long-lasting response to cisplatinum in recurrent malignant thymoma. Cancer 49:1985–1987, 1982.

BRIEF REPORTS

Phase I Evaluation of β-2′-Deoxythioguanosine in Pediatric Patients With Leukemia[1,2]

Gussie R. Higgins,† Denise C. Jamin, Nomie A. Shore,* Richard Momparler, Gary Hartman, and Stuart E. Siegel[3]

Thirty-one pediatric patients with acute leukemia who had relapsed on either 6-mercaptopurine or 6-thioguanine were treated with β-2′-deoxythioguanosine, which was administered as an iv infusion every 12 hours for three or six doses every 2 weeks. Severe nausea and vomiting and urate nephropathy were the dose-limiting toxic effects. Therapeutic responses occurred in four of 24 children with acute lymphocytic leukemia and in two of seven with acute nonlymphoblastic leukemia. [Cancer Treat Rep 69:699–701, 1985]

The purine analog β-2′-deoxythioguanosine (β-TGdR) was developed to circumvent resistance to 6-thioguanine (6-TG) (1–3). Conflicting experimental data exist as to whether β-TGdR has significant activity against 6-TG-resistant experimental tumors (4,5).

Clinical studies have indicated that β-TGdR is active in patients with acute leukemia who had previously received other purine analogs (6,7). The present study was designed to determine the toxic effects and the maximum tolerated dose of β-TGdR in children with acute leukemia.

MATERIALS AND METHODS

Thirty-one pediatric patients with acute leukemia who had been treated previously with either 6-mercaptopurine (6-MP) or 6-TG received β-TGdR.

The baseline and follow-up evaluations included bone marrow examinations, history and physical examinations, and weekly cbcs, chemistries (creatinine, BUN, uric acid, bilirubin, total protein, SGOT, SGPT, alkaline phosphatase, calcium, phosphorus, and amylase), and urinalysis. Allopurinol was given to patients with wbc counts $> 15{,}000/\text{mm}^3$ and to patients with serum uric acid levels > 9.0 mg/dl. It was not given routinely to all patients because allopurinol does not interfere with the metabolism of β-TGdR (8). In the absence of blasts in the peripheral blood, a bone marrow examination was performed prior to each course of therapy. Response to therapy was evaluated according to criteria of the Childrens Cancer Study Group (9,10).

β-TGdR was supplied as a lyophilized powder in 200-mg vials by the Clinical Drug Evaluation Branch, National Cancer Institute, Bethesda, MD. The drug was reconstituted with 9.9 ml of sterile water, diluted with 5% dextrose in 0.2% saline to a concentration of 2–4 mg/ml, and administered by iv infusion over 2–4 hours. The scheduled administration was every 12 hours for three or six doses. Courses were repeated at 2-week intervals provided recovery from toxic side effects had occurred. The initial dose was 200 mg/m^2. This dose was increased in accordance with a modified Fibonacci schema to a maximum dose of 2250 mg/m^2 (11). Dosage escalations were permitted in an individual patient in the absence of toxicity during the previous course. Twenty-five patients received two or more courses of therapy.

RESULTS

Reversible urate nephropathy, elevations of liver enzymes, nausea and vomiting, alopecia, and skin reactions were observed (table 1). Elevated serum uric acid occurred in only five children, all of whom had received doses $\geqslant 1500$ mg/m^2. Maximum uric acid levels ranged from 9.2 to 14.7 mg/dl and occurred on the second or third day of therapy. The uric acid values returned to normal within 5 days of the maximum level. Values for serum creatinine, ranging from 1.3 to 2.5 mg/dl, were associated with the increases in serum uric acid.

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[3] Division of Hematology-Oncology, Childrens Hospital of Los Angeles, and University of Southern California School of Medicine.

†Deceased.

**Reprint requests to:* Nomie A. Shore, MD, Division of Hematology-Oncology, Childrens Hospital of Los Angeles, PO Box 54700, Los Angeles, CA 90054.

TABLE 1.—β-TGdR toxicity and response in 31 pediatric patients (26 boys, 5 girls)

Dose (mg/m^2 q 12 hrs) × No. of doses	Total No. of patients at dose level	No. of patients with toxic effects					No. of patients with therapeutic response*
		Renal	Hepatic	Gastro-intestinal	Alopecia	Skin	
< 400 × 6	7	0	1	0	6	0	1 (CR, ALL)
400 × 6	2	0	0	0	2	1	0
450 × 6	6	0	2	0	6	2	1 (PR, ANLL)
750 × 3	8	0	2	2	0	0	1 (PR, ALL)
1000 × 3	9	0	0	3	0	0	1 (CR, ALL)
1250 × 3	6	0	0	5	0	0	1 (PR, AML)
1500 × 3	4	0	1	4	0	0	0
1750 × 3	11	3	2	11	0	0	1 (PR, ALL)
2000 × 3	6	1	3	6	0	0	0
2250 × 3	2	1	0	2	0	0	0

*CR = complete remission; ALL = acute lymphocytic leukemia; PR = partial remission; ANLL = acute nonlymphoblastic leukemia; and AML = acute myelogenous leukemia.

Increased liver enzymes occurred in 11 children (table 1). Maximum SGOT values ranged from 50 to 925 units/L; maximum SGPT values ranged from 38 to 780 units/L. Liver enzymes returned to normal in five of the 11 patients. In the remaining six patients, all with progressive leukemia, enzymes increased after therapy and remained elevated until time of death. Post-mortem hepatic histopathologic examinations performed in three of these six patients showed leukemic cell infiltration, focal necrosis, and monilial granulomata (one patient).

Vomiting was related more to individual dose level than to total dose per course and was uncommon at doses < 1250 mg/m^2. Dose-limiting toxicity due to severe, uncontrollable vomiting occurred at 2250 mg/m^2.

Three patients developed generalized erythematous macular rashes within 7-10 days following β-TGdR administration. The rashes persisted for 3-10 days and recurred in one but not in the other two patients following subsequent β-TGdR administration. Myelosuppression, which was difficult to evaluate in patients with leukemia, was defined as a pretreatment absolute neutrophil count $\geqslant 1000/mm^3$, falling by ⩾ 25% after treatment, and then rising to $\geqslant 1000/mm^3$ prior to the next course of therapy. Only three patients at dose levels of 200 mg/m^2 for six doses, 750 mg/m^2 for three doses, and 1500 mg/m^2 for three doses fulfilled these criteria. Therefore, myelosuppression was not used to evaluate drug toxicity.

Therapeutic Response

Six therapeutic responses were documented (table 1). Two patients with acute lymphocytic leukemia and one with acute nonlymphoblastic leukemia achieved complete remission. All responses were observed at doses ⩽ 1750 mg/m^2 and occurred after one to six courses.

DISCUSSION

The initial schedule of administration every 12 hours for six doses was selected based on mouse data, which showed that efficacy was directly related to duration of therapy (12). In the phase I study, when a dose of 450 mg/m^2 every 12 hours for six doses had been reached, no dose-limiting toxicity was observed. Two patients who had achieved remissions were unable to tolerate maintenance therapy at this dose because of evaluable hematopoietic toxicity.

The six-dose regimen was shortened to a three-dose schedule because dose-limiting myelosuppression had been reported in phase I studies of adults who received 500 mg/m^2 daily for 5 days (6) and because additional mouse data from our laboratory suggested that higher doses could be given with less toxicity if the duration were shortened.

Severe nausea and vomiting, uncontrolled by antiemetics, was the dose-limiting toxicity in this study. The maximum tolerated dose was 1750 mg/m^2 given every 12 hours for three doses every 2 weeks.

Abnormalities in liver function, rashes, and alopecia were similar in severity to those observed in other clinical trials (6,7).

Acute reversible renal failure (elevated serum creatinine) was observed in association with hyperuricemia at doses ⩾ 1750 mg/m^2. Although hyperuricemia could be attributed to endogenous purine degradation, the possibility exists that β-TGdR catabolism may have contributed to the elevation of serum uric acid, since approximately 28% of β-TGdR is metabolized to thiouric acid (8).

Although six therapeutic responses occurred in the 31 patients with acute leukemia, it is possible that not all patients had disease refractory to antipurines and that a similar therapeutic effect may have occurred had equivalent doses of 6-TG or 6-MP been given to these patients.

Further studies should be done to determine if β-TGdR is effective in patients refractory to other antipurines.

REFERENCES

1. BROCKMAN RW. Symposium on the experimental pharmacology and clinical use of antimetabolite analogs with anticancer activity. Clin

Pharmacol Ther 2:237-261, 1961.

2. STUTTS P, and BROCKMAN RW. A biochemical basis for resistance of L1210 mouse leukemia to 6-thioguanine. Biochem Pharmacol 12:97-104, 1963.
3. ELLIS DB, and LEPAGE GA. Biochemical studies of resistance to 6-thioguanine. Cancer Res 23:436, 1963.
4. LEPAGE GA, JUNGA IG, and BOWMAN B. Biochemical and carcinostatic effects of 2′-deoxythioguanosine against two tumors resistant to 6-thioguanine. Cancer Res 35:1372-1374, 1975.
5. NELSON JA, KUHNS JN, and CARPENTER JW. Lack of activity of β-2′-deoxythioguanosine against two tumors resistant to 6-thioguanine. Cancer Res 35:1372-1374, 1975.
6. BODEY GP, MCCREDIE KB, WHITECAR JP, ET AL. Clinical studies of β-thioguanine deoxyriboside alone and in combination with arabinosyl cytosine. Med Pediatr Oncol 2:199-205, 1976.
7. OMURA GA, VOGLER WR, SMALLEY RV, ET AL. Phase II study of β-2′-deoxythioguanosine in adult acute leukemia. Cancer Treat Rep 61:1379-1381, 1977.
8. LU K, BENVENUTO JA, BODEY GP, ET AL. Pharmacokinetics and metabolism of β-2′-deoxythioguanosine and 6-thioguanine in man. Cancer Chemother Pharmacol 8:119-123, 1981.
9. PIERCE M, SHORE N, SITARTZ A, ET AL. Cyclophosphamide therapy in acute leukemia of childhood. Cancer 19:1551-1560, 1966.
10. CHARD R, FINKLESTEIN J, SONELY M, ET AL. Increased survival in childhood acute non-lymphoblastic leukemia after treatment with prednisone, cytosine arabinoside, 6-thioguanine, cyclophosphamide, and Oncovin (PATCO). Med Pediatr Oncol 4:263-273, 1978.
11. CARTER SK. Clinical trials in cancer chemotherapy. Cancer 40:544-557, 1977.
12. MOMPARLER RL, HIGGINS GR, SIEGEL SE, ET AL. Antileukemic therapy and toxicology studies in mice with β-2′-deoxythioguanosine. Cancer Treat Rep 62:135-137, 1978.

Phase I Study of Bisantrene in Acute Nonlymphoblastic Leukemia[1,2]

M. Marty,[3,*] C. Ferme,[3] C. Gisselbrecht,[3] H. Guy,[4] M. J. Clark,[3] A. Bancillon,[3] and M. Boiron[3]

A phase I study of bisantrene using a daily injection for 5 days was undertaken in adult patients with relapsing acute nonlymphoblastic leukemia. Seventeen patients received 27 courses, with daily doses ranging from 75 to 250 mg/m^2. Although gastrointestinal toxicity and alopecia were rare, hematological toxicity occurred in 85% of the patients. There was cholestasis unrelated to infectious events in 27% of the courses, as well as reversible renal failure in eight of 27 evaluable courses. Responses (complete + partial) were obtained in 35% ± 10% of the patients. The recommended dose for phase II study is 200 mg/m^2/day × 7. [Cancer Treat Rep 69:703–705, 1985]

Bisantrene is an anthracene derivative with antitumor activity similar to that of doxorubicin in experimental mouse tumor systems (1). Experimental studies with human tumor cloning assay (2) have suggested wide antitumor activity. Possible liver and renal toxicity (3) has been suggested by animal experimentation. In vitro and in vivo pharmacological studies have suggested superiority of bisantrene over mitoxantrone, since bisantrene shows higher maximum dose tolerance, longer terminal half-life, and less important plasma-protein binding (4). In previous phase I trials, leukopenia and phlebitis were found to be the dose-limiting toxic effects (5,6). We therefore initiated a phase I trial using the recommended schedule of 75 mg/m^2/day × 5 (7) as a starting dosage for patients with acute nonlymphoblastic leukemia (ANLL) to determine the optimal schedule for this disease.

PATIENTS AND METHODS

Seventeen adult patients (seven male, ten female) with relapsed ANLL received 27 courses of bisantrene; their median age was 39 years. All patients except one had ANLL in first relapse (three had M_1; five, M_2; three, M_3; one, M_4; three, M_5; and one, acute undifferentiated leukemia). The mean duration of first complete response (CR) was 9 months, significantly shorter than the 16-month median duration of first CR in current protocols (8). These patients had previously received an average of eight antileukemic agents: all had received cytarabine (mean dose = 6000 mg/m^2); anthracycline plus either daunorubicin (DNR) or zorubicin (mean dose = 550 mg/m^2 of DNR or equivalent); or 6-mercaptopurine or 6-thioguanine (mean dose = 1750 mg/m^2).

Relapse was documented by bone marrow aspirate showing > 25% leukemic cells in a normal or hypercellular marrow. Criteria for eligibility were normal cardiac function, bilirubin < 1.5 mg/dl; creatinine < 1.5 mg/dl, and absence of antileukemic therapy for at least 4 weeks prior to entry in the study. Bisantrene was given as a 2-hour daily infusion for 5 days [through central venous (Vygon) catheter in 16 of 17 patients]. The initial starting daily dose was 75 mg/m^2; it was increased by 25 mg/m^2 every three courses. A second course was given in the event of persisting leukemic cells in blood or bone marrow and/or tumor at Day 15. All were inpatients and received symptomatic therapy including antibiotics and transfusions when necessary. The number of courses at each dose level is summarized in table 1. Two courses could not be evaluated for antileukemic effect because of early death due to septicemia (Days 6 and 7).

RESULTS

Tolerance

Tolerance was evaluable for all patients after the first course. Fifteen patients (88%) showed dose-related hematological toxicity. The mean wbc and platelet count nadirs occurred on Days 8–9 (range, 4–21 days). The mean duration of leukopenia and thrombocytopenia was

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[2] Supported by INSERM-NCI agreement.

[3] Institut de Recherches sur les Leucémies et les Maladies du Sang, Hôpital Saint-Louis, Paris, France.

[4] Unité d'Hématologie, Clinique Médicale, Hôpital du Bocage, Dijon, France.

[*] *Reprint requests to:* Dr. M. Marty, Institut de Recherches sur les Leucémies et les Maladies du Sang, Hôpital Saint-Louis, 2 place du Docteur Alfred Fournier, 75475 Paris Cedex, France.

TABLE 1.—Dose levels and No. of courses (dose/m²/day × 5)

Dose level (L) (mg/m²/day)	No. of courses (L × 5)	No. of evaluable courses
75	3	3
100	3	3
125	3	2
150	3	3
175	3	3
200	6	5
225	3	3
250	3	3
Total	27	25

10 days (range, 4-22). The mean wbc count nadir was 700/μl (range, 200-3000); the mean platelet count nadir was 35,000/μl (range, 10,000-80,000). Despite difficulties in grading hematological toxicity in leukemic patients, the bisantrene toxicity grade did not appear to be more pronounced than for any major antileukemic agent.

Alopecia was seen in four of 16 evaluable patients. Nausea and vomiting were induced in four of 17 patients (23%); they were always moderately severe (grades 1-2). Phlebitis was prevented in 16 patients by the use an iv catheter. Renal and liver toxic effects were moderately frequent. Renal toxicity occurred in eight of 27 courses. It was documented by a serum creatinine increase (mean maximum value, 2 mg/dl; range, 1.3-3.7) without urinary flow reduction, proteinuria, or hematuria and appeared between Days 12 and 13. All signs reverted to normal within 5-20 days. Renal toxicity was dose-related and was not observed for cumulative doses < 1250 mg/m². Prevention was not attempted by forced diuresis and/or fractionated administration of bisantrene. In this study, we could not assess the role of previously received nephrotoxic agents.

Hepatic toxicity occurred in six of 26 evaluable courses. Its onset was 15-25 days after initiation of bisantrene. Clinical and biological findings were suggestive of cholestasis, with increased serum bilirubin (mean maximum value, 3 × normal), alkaline phosphatase (mean maximum value, 3.3 × normal), and, to a lesser extent, SGOT (mean value, 2.3 × normal). No concurrent viral infection (hepatitis B or cytomegalovirus) could be demonstrated in those patients. None received other drugs known for their liver toxicity. Pathological findings in three patients showed cholestasis without cytolysis, inflammatory or leukemic cell infiltration, or vascular abnormalities. We observed no signs of liver failure and all abnormalities disappeared within 2 months in three evaluable patients. Allergic mechanism did not seem to be involved, since two of these patients were rechallenged with bisantrene without further liver abnormality.

Antileukemic Effects

Four CRs (23% ± 10% of the patients), two partial responses (PRs), four bone marrow responses, and four minor responses were observed. A significant response rate (CR + PR) was achieved in 35% ± 10% of the patients. Response was dose-related, since three CRs were achieved after the first course of bisantrene at a cumulative dose > 1000 mg/m² (1250, 1500, 1500). Remission was maintained by monthly courses of bisantrene given as a single injection and has been of short duration (4, 5, 5, and 6 months).

DISCUSSION

This phase I study of bisantrene in adult patients with relapsing ANLL has shown that significant antileukemic effect could be achieved with acceptable although significant toxicity. Digestive and hematological toxic effects were less pronounced than with other single-agent therapy (zorubicin, Ara-C, 5-azacitidine, or amsacrine). Doses could be increased to 250 mg/m²/day × 5, ie, four times the initially planned dose for this schedule. However, we observed renal and liver toxic effects that were not encountered in previous studies. Renal toxicity seemed to be related mostly to tubular damage. It appeared early and was of short duration (10 days). It was of limited severity (grades 2-3). We did not observe renal abnormalities in patients receiving 1250-1700 mg/m² given in two courses, which suggests that increased fractionation of the dose could prevent this toxic effect.

Liver toxicity was observed in 27% of the evaluable courses. It was biologically and pathologically related to the cholestasis with delayed onset and was totally reversible in three patients. While this toxic effect might be dose-limiting, its mechanism remains unclear. It could be related to direct cytotoxicity. The 30% occurrence of major responses in heavily pretreated patients is encouraging. We therefore conclude that antileukemic effect can be achieved with acceptable toxicity; further phase II studies are therefore warranted. The recommended cumulative dose administered in one course is 1400 mg/m². Since there is evidence that dose fractionation could cancel or lessen renal toxicity, a 200-mg/m²/day × 7 schedule should be studied. Such a schedule is currently under study in patients with advanced acute lymphoblastic leukemia, ANLL, and blastic crisis of chronic myeloblastic leukemia.

REFERENCES

1. CITARELLA RV, WALLACE RE, MURDOCK KC, ET AL. Activity of a novel anthracenylbishydrazone, 9,10-anthracenedicarboxyaldehyde bis [(4,5-dihydro-1*H*-imidazol-2-yl)hydrazone] dihydrochloride, against experimental tumors in mice. Cancer Res 42:440-444, 1982.
2. COWAN JD, VON HOFF DD, CLARK GM, ET AL. Comparative human tumor sensitivity to adriamycin (A), mitoxantrone (M), and bisantrene (B) as measured by a human tumor cloning assay (HTCA). Proc Am Assoc Cancer Res 23:734, 1982.
3. DURR EE. Preclinical toxicology studies on mitoxantrone and bisantrene. *In* Proceedings of the Third NCI-EORTC Symposium on New

Drugs in Cancer Therapy, Brussels, European Organization for Research on Treatment of Cancer, Oct 15-17, 1981.

4. ALBERTS DS, PENG YM, and DAVIS TP. Comparative pharmacology of mitoxantrone and bisantrene. Cell Tissue Kinet 15:199, 1982.
5. VON HOFF DD, MYERS JW, KUHN J, ET AL. Phase I clinical investigation of 9,10-anthracenedicarboxyaldehyde bis [(4,5-dihydro-1*H*-imidazol-2-yl)hydrazone] dichloride. Cancer Res 41:3118-3121, 1981.
6. ALBERTS DS, MACKEL C, POCELINKO R, ET AL. Phase I clinical investigation of 9,10-anthracenedicarboxaldehyde bis [(4,5-dihydro-1*H*-imidazol-2-yl)hydrazone] dihydrochloride with correlative in vitro human tumor clonogenic assay. Cancer Res 42:1170-1175, 1982.
7. SPIEGEL RJ, BLUM RH, LEVIN M, ET AL. Phase I clinical trial of 9,10-anthracene dicarboxaldehyde (bisantrene) administered in a five-day schedule. Cancer Res 42:354-358, 1982.
8. MARTY M, FERME C, SCHAISON G, ET AL. 16LA79 protocol in A.N.L.L.: therapeutic value of high dose Ara-C-rubidazone in remission induction regimen. *In* Proceedings of the 3rd International Symposium on Therapy of Acute Leukemias. Rome, Dec 11-14, 1982.

Phase II Study of Elliptinium in Advanced Breast Cancer[1]

J. G. Rouësse,[2,*] T. Le Chevalier,[2] P. Caille,[3] J. M. Mondesir,[3] H. Sancho-Garnier,[2] F. May-Levin,[2] M. Spielmann,[2] R. De Jager,[3] and J. L. Amiel[2,4]

A group of 74 patients with advanced breast cancer received elliptinium as second- or third-line treatment (100 mg/m²/week). The objective response rate was 19% (30% in soft tissue metastases), lasting from 3 to 12 months. This drug appears to have no marrow toxicity. Mild to moderate nausea and mouth dryness were the most frequently encountered side effects. Hemolysis occurred in five patients who had an IgM antibody and represents the dose-limiting toxicity. Cumulative renal toxicity (World Health Organization, grade 2) was observed in one of ten patients who had received > 2000 mg of elliptinium. [Cancer Treat Rep 69:707–708, 1985]

The antitumor activity of ellipticines correlates with their affinity for DNA, although intercalation may only be one of the potential mechanisms of action. Elliptinium was selected for clinical trial on the basis of its broad spectrum of activity in the antitumor screen (1).

Therapeutic activity against breast cancer was detected during phase I studies (2), and a weekly schedule at a dose of 80-100 mg/m² was advocated for phase II clinical trials.

PATIENTS AND METHODS

Between June 1979 and June 1982, 74 postmenopausal patients with advanced breast cancer who were not candidates for hormonal therapy received elliptinium as second- or third-line treatment. All of the patients had received prior combination chemotherapy, including doxorubicin (81% of patients), vincristine (81%), methotrexate (78%), cyclophosphamide (69%), and 5-FU (68%). Age ranged from 28 to 76 years (median, 54). Hormonal receptors were known in 14 patients [three were estrogen receptor (ER)-positive and progesterone receptor (PR)-positive; four were ER-positive and PR-negative; and seven were ER-negative and PR-negative]. Eligibility criteria included performance status (Eastern Cooperative Oncology Group) of 0-3, documented progression of disease, no prior hormonal therapy within 6 weeks, no prior chemotherapy or radiation therapy within 4 weeks, recovery from toxicity induced by prior treatment, granulocyte count > 1500/mm³, platelet count > 50,000/mm³, serum creatinine < 1.1 mg/dl, and serum bilirubin < 2 mg/dl. All patients had measurable disease and informed consent was obtained according to institutional regulations.

Elliptinium was supplied by Sanofi Recherche, Paris, France, as a sterile lyophilized orange powder. The drug was dissolved in 250 ml of 5% glucose solution and administered as a slow iv infusion over 60-90 minutes. Patients received 100 mg/m² once a week for 6 weeks. Thereafter, the interval between injections was increased to every 2-4 weeks according to the individual tolerance of the patients. The first response evaluation was done 4 weeks after starting elliptinium, when patients had received a minimum of three weekly doses. Treatment was continued in patients where objective tumor regression or stabilization of disease was observed.

The World Health Organization criteria of response were used for evaluation of response and toxicity (3).

RESULTS

Of the 74 patients in the study, 11 were considered inevaluable for response because of early death that was disease-related (two patients), treatment refusal (two), loss to follow-up (two), and toxicity before 4 weeks after starting (five).

Of 63 evaluable patients, complete response (CR) was achieved in two patients and partial response (PR) was

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[2] Institut Gustave-Roussy, Villejuif, France.

[3] Sanofi Recherche, Paris, France.

[4] We thank Dr. Frederick P. Smith, Georgetown University Hospital, Washington, DC, for his editorial advice.

[*] *Reprint requests to:* J. G. Rouësse, MD, Institut Gustave-Roussy, Rue Camille Desmoulins, 94805 Villejuif Cedex, France.

TABLE 1.—Response

Target	No. of patients					PR + CR
	Total	Failure	MR*	PR	CR	(%)
Skin	36	15	9	8	4	33
Lymph node	11	3	6		2	18
Pleural	11	3	7	1		9
Lung	17	14	2	1		5
Liver	10	8	2			
Abdominal	1				1	

*MR = minor response.

achieved in ten. The objective response rate was 19%. Minor responses were observed in ten patients. Mean duration of response was 22 weeks (range, 10–53).

Forty-three patients had more than one site involved. Elliptinium seems to have a selective activity on cutaneous or subcutaneous and lymph node metastases: response rates were 33% and 18%, respectively, for these sites, in contrast to 9% and 5% for pleural and lung metastases (table 1).

Toxicity

The side effects of elliptinium are summarized in table 2. The lack of marrow toxicity must be emphasized. Acute hemolysis was observed in five patients. An indirect agglutination test with elliptinium was performed sequentially before each treatment in 18 patients. Acute hemolysis occurred in four of nine patients with a positive test. No hemolysis was observed in patients without antibodies. The presence of IgM antibody was correlated with clinical hemolysis. Treatment was stopped in all patients with hemolysis, in four with dryness of mouth, in two with dryness of mouth and cramps, and in three with periphlebitis. In five patients treatment was stopped before evaluation of response.

Unreversible renal toxicity manifested by increase in serum creatinine (World Health Organization, grade 2) was observed in one of ten patients who had a cumulative dose of elliptinium > 2000 mg. There was no nephrotoxicity in patients receiving less than this cumulative dose with this weekly schedule.

TABLE 2.—Side effects

Side effect	No. of patients (%)
Nausea	58 (78)
Severe vomiting	22 (30)
Severe dryness of mouth	24 (32)
Phlebitis	9 (12)
Cramps	8 (11)
Hemolysis	5 (7)
Fever, shiver	3 (4)
Renal toxicity	1 (1.3)

DISCUSSION

Elliptinium is an active agent for metastatic breast cancer (4). Even after refractoriness to prior chemotherapeutic regimens, objective responses were obtained in 12 of 63 evaluable patients with our weekly schedule of 100 mg/m^2.

The lack of alopecia and myelotoxicity deserves emphasis; this should allow the incorporation of elliptinium into combinations where myelosuppression represents the dose-limiting toxic effect. The major side effects were dryness of mouth and phlebitis.

Hemolysis was the dose-limiting toxic effect in our study. In other trials (5), we documented Coombs' positive reactions in 30 of 146 patients (20.5%); while hemolysis was observed in ten of these patients (6.8% of the total), no hemolysis was detected in the remaining 116 patients. This immunogenicity of the drug appears to be schedule-dependent: patients who were treated for 3 consecutive days every 3 weeks had no positive agglutination tests and no hemolysis. We have initiated new studies with this latter schedule.

REFERENCES

1. LE PECQ JB, DAT-XUONG N, GOSSE C, ET AL. A new antitumoral agent: 9-hydroxy-ellipticine. Possibility of a rational design of anticancerous drugs in the series of DNA intercalating drugs. Proc Natl Acad Sci USA 71:5078–5082, 1974.
2. JURET P, TANGUY A, GIRARD A, ET AL. L'acétate d'hydroxy-9-methyl-2-ellipticinium (NSC 264-137). Etude toxicologique et thérapeutique chez 100 cancéreux. Nouv Presse Med 8:1495–1498, 1979.
3. WHO Handbook for Reporting Results of Cancer Treatment. Geneva, WHO Offset Publication, 1979, vol 48, pp 22–24.
4. ROUESSE J, TURSZ T, LE CHEVALIER T, ET AL. Intérêt de la 2 *N*-méthyl-9-hydroxy-ellipticine (NSC 264-137) dans le traitement des cancers métastasés. Nouv Presse Med 10:1997–1999, 1981.
5. MONDESIR JM, DUCRET JP, KRAMAR A, ET AL. Elliptinium acetate (9-hydroxy-2-methyl elliptinium acetate, NMHE) induced hemolysis. *In* Proceedings of the 8th Annual Meeting of European Society for Medical Oncology. Nice, Springer International, 1982, p 40.

Phase II Trial of Diaziquone in Advanced Large Bowel Carcinoma in Previously Treated and Untreated Patients: A Southwest Oncology Group Study[1,2]

Richard Shildt,[3] Ronald L. Stephens,[4] V. P. Subramanian,[5] Laurence H. Baker,[6] William S. Fletcher,[7] Robert M. O'Bryan,[8] and Joseph D. McCracken[9,*]

Diaziquone (AZQ) is a bifunctional quinone structually similar to mitomycin and nitrosoureas. The presumed mechanism of its antineoplastic activity is alkylation of DNA. Phase I studies by Krakoff[10] reported that myelosuppression, nausea, and vomiting were the limiting toxic effects and that an appropriate starting dose was 7 mg/m^2 daily for 5 days. Since AZQ is structually similar to other agents with antineoplastic activity against colon carcinoma, a phase II trial was initiated. The study was designed to determine the therapeutic value of AZQ in patients with metastatic colon cancer.

PATIENTS AND METHODS

Ninety-one patients with metastatic adenocarcinoma of the colon were entered in this trial. Twenty-two patients from a previous phase II trial who had been treated with 5-FU were crossed over to AZQ in this study. Eligibility requirements included: clinically measurable disease; a life expectancy of at least 10 weeks; a performance status of at least 2 (Zubrod performance scale); a wbc count of at least 4000/mm^3; and a platelet count of at least 100,000/mm^3. A signed informed consent was obtained from all patients entered in the study. Simultaneous radiotherapy was permitted as long as the measurable lesion was not being irradiated. Patient characteristics are shown in table 1.

The starting dose of AZQ was 7 mg/m^2 iv bolus for 5 days, repeated every 28 days as long as all toxic effects had cleared. If the granulocyte count nadir remained $> 4000/\text{mm}^3$ and the platelet count remained $> 100{,}000/\text{mm}^3$, the daily dose was increased to 9 mg/m^2. If the wbc count nadir was $< 2000/\text{mm}^3$ or the platelet count nadir was $< 25{,}000/\text{mm}^3$, a dose reduction to 4 mg/m^2/day was permitted. An adequate trial was considered a 5-day course of AZQ with a follow-up period of at least 23 days.

Complete remission (CR) was defined as the disappearance of all clinically recognizable evidence of tumor for 4 weeks. A decrease by at least 50% in the sums of the products of the perpendicular diameters of measurable sites was the criterion for a partial remission (PR). Patients with no change in tumor measurements for at least 4 months were classified as having stable disease. Progressive disease was defined as an increase of $\geqslant 25\%$ in the sum of the products of the perpendicular diameters of measurable sites or the development of new metastatic sites.

RESULTS

Ninety-one patients with metastatic colon cancer were entered into the study. Seventy-five patients were evaluable (53 previously untreated and 22 previously treated with 5-FU). Sixteen patients were not evaluable (seven who died early, six who were lost to follow-up or had inadequate data, and three who refused therapy). In the previously untreated group of patients, there was one CR, seven with stable disease, and 45 with progressive disease. One patient achieved CR status after 10 months of AZQ and remains in CR after another 11 months. The computerized axial tomographic scan of the liver showed two 2-cm lesions which disappeared, and the CEA dropped from 48 to 26. A normal CEA level was not a criterion in the study for a CR. In the group of patients previously treated with 5-FU, there were no CRs or PRs, three with stable disease, and 19 with progressive disease.

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[3] University of Oklahoma Health Sciences Center, Tulsa.

[4] University of Kansas Medical Center, Kansas City.

[5] Olin E. Teague Veteran's Center at Scott and White, Temple, TX.

[6] Wayne State University Medical Center, Detroit, MI.

[7] University of Oregon Health Sciences Center, Portland.

[8] Henry Ford Hospital, Detroit, MI.

[9] Brooke Army Medical Center, San Antonio, TX.

[10] Krakoff I. Minutes of the Phase I Working Group, Dec 2, 1980.

* *Reprint requests to:* Southwest Oncology (SWOG 7945), Operations Office, 4450 Medical Dr, San Antonio, TX 78229.

TABLE 1.—Patient characteristics*

	Previously untreated	Prior 5-FU
Total evaluable	53	22
Age (yrs)		
Median	63	62
Range	28-75	33-75
Sex		
Male	33	12
Female	20	10
Performance status		
0	17	4
1	24	10
2	12	8
Dose increases	17	4
Dose decreases	9	3
Wbc toxicity (No. of cycles)		
Grade 1-2 (2000-4000/mm^3)	15	6
Grade 3-4 ($< 2000/mm^3$)	11	3
Response		
Complete	1	0
Partial	0	0
Stable	7	3
Progression	45	19
No. of cycles/patient		
1-3	39	17
4-6	12	3
13	2	2
CEA (ng/ml)		
Median	24	159
Range	1.4-11,000	2.1-2500
LDH		
Median	249	168
Range	79-1189	23-1172

* Unless otherwise indicated, values = No. of patients.

The major toxic effect was marrow toxicity. The majority of the reported granulocyte count nadirs fell between 2000 and 4000/mm^3. Thrombocytopenia was seen, but much less than neutropenia. Nausea and vomiting of a mild nature was the second most common toxicity. There were no other side effects of significance reported and there were no drug-related deaths.

DISCUSSION

Only one of 75 patients who received AZQ in this study had a documented response (~ 1%). In a previous study, patients with colon carcinoma were given AZQ at 22.5 mg/m^2 once every 4 weeks (1). Only one PR, lasting 59 days, was reported in 31 treated patients. Toxicity in both studies was similar, in that myelosuppression was the most common side effect, and the degree of suppression was mild. AZQ, as used in this study, did not appear to have clinically significant antitumor effects against colon carcinoma.

Presently, for all phase II studies in the Southwest Oncology Group, registration is temporarily halted at 20 patients. If review of toxicity and response data is favorable, the study will be reopened for a specified number of patients. This procedure will ensure earlier closing of phase II studies with inactive drugs.

REFERENCE

1. RUBIN J, VANHAZEL GA, SCHUTT HA, ET AL. Phase II study of aziridinylbenzoquinone (AZQ) in advanced large cell carcinoma. Am J Clin Oncol 5:539-540, 1982.

Phase II Trial of High-Dose Cisplatin in Patients With Malignant Mesothelioma[1,2]

David M. Mintzer,[3] David Kelsen,[3,*] Daniel Frimmer,[4] Robert Heelan,[3] and Richard Gralla[3]

Twenty-five patients with advanced malignant mesothelioma were treated in a phase II trial with high-dose cisplatin (DDP). All patients had measurable or evaluable disease. Seven patients had received prior chemotherapy. DDP (120 mg/m^2) with mannitol and prehydration was given every 4 weeks for two doses and then every 6 weeks. Of 24 patients evaluable for response, three had partial responses (3, 3+, and 9 months; response rate, 13%; 95% confidence limits, 4%–31%) and one had minor response. All responses occurred in previously untreated patients. High-dose DDP has modest activity in malignant mesothelioma. [Cancer Treat Rep 69:711–712, 1985]

Malignant mesothelioma is an aggressive neoplasm which, despite present therapies, is usually associated with unrelenting disease progression, with median survival times of < 2 years (1-6). Because of the lack of effective treatment for this disease, as well as the anticipated increase in incidence over the next decades, there is a clear need for new and effective therapies. In this report, we describe results with high-dose cisplatin (DDP) in the treatment of malignant mesothelioma.

MATERIALS AND METHODS

Twenty-five patients with advanced malignant mesothelioma were treated from April 1981 to March 1984. In 24 patients, the diagnosis was confirmed by review of relevant pathologic material by the Department of Pathology, Memorial Sloan-Kettering Cancer Center. In the remaining patient, the slides were reviewed by two independent outside pathologists. Requirements for entry included a performance status (Karnofsky) ⩾ 50%, age < 75 years, creatinine clearance > 65 ml/minute, clinically normal auditory function, New York Heart Association class I or II, wbc count > 3000/μl, and platelet count > 100,000/μl. Patients must not have received irradiation or chemotherapy within 4 weeks of starting treatment. Informed consent was obtained from all patients prior to the initiation of therapy.

Twenty-three of the patients were men and two were women. The median age was 61 years (range, 45-74); the median performance status was 70% (range, 50%-100%). Twenty-four patients had pleural mesothelioma and one had peritoneal mesothelioma. Of those patients, 13 had epithelial mesothelioma, three had fibrosarcomatous mesothelioma, and seven had mixed histology. Sixteen patients reported a history of asbestos exposure. Prior treatment included chemotherapy (seven patients) and pleurectomy plus irradiation (nine patients). All patients had measurable or evaluable disease: three had measurable disease on physical examination, one had measurable disease on chest x-ray, and 21 had evaluable disease on chest x-ray or computerized tomographic scan.

All patients were treated with high-dose DDP (120 mg/m^2) over a 20-minute infusion with mannitol and prehydration using the method of Hayes et al (7). DDP was given every 4 weeks for two doses and then every 6 weeks. This schedule was selected to minimize chronic toxicity. All patients received antiemetic therapy, generally with high-dose metoclopramide combination regimens (8).

A complete remission was defined as 100% disease regression for at least 1 month. For patients with measurable disease, a partial remission was defined as at least a 50% but < 100% decrease in the product of the two longest perpendicular diameters of clearly measurable lesions for at least 1 month. A minor regression was defined as a > 25% but < 50% decrease in tumor size. For patients with evaluable disease a reference radiologist (R. Heelan) was used to score responses. In all patients, no new le-

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[3] Solid Tumor Service, Department of Medicine, and the Department of Diagnostic Radiology, Memorial Sloan-Kettering Cancer Center, and Department of Medicine, Cornell University Medical College, New York, NY.

[4] Muhlenberg Hospital, Plainfield, NJ.

[*] *Reprint requests to:* David Kelsen, MD, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021.

sions could appear during the remission period. If there was no change in the size of the lesions, the patient was considered to be a nonresponder.

RESULTS

Of 25 patients treated, 24 were evaluable for response. One patient was considered inevaluable because he received another agent along with DDP. There were three partial responses (3, 3+, and 9 months; 13% response rate; 95% confidence limits, 4%-31%) and one minor response. All responses occurred in previously untreated patients, all had pleural epithelial-type primaries, and all had a performance status of at least 60%. The median survival of evaluable patients was 5 months (range, 1-22+).

Toxic effects included nephrotoxicity, with a median peak creatinine of 1.2 mg/100 ml (range, 0.8-3.9) and creatinine > 2 mg/100 ml in four patients. Myelosuppression was modest, with a median wbc count nadir of 5600/mm^3 (range, 1900-34,000) and a median platelet count nadir of 297,000/mm^3 (range, 18,000-709,000). Peripheral neuropathy required discontinuation of treatment in one patient. Treatment in one partial responder was discontinued after 3 months because of severe nausea and vomiting. There were no drug-related deaths.

DISCUSSION

Adequate chemotherapeutic trials in mesothelioma have been limited because of the relative rarity of the tumor as well as the difficulty in assessing therapeutic responses (5). Over the past several years, however, use of computerized tomography has improved the ability to evaluate intrathoracic or abdominal disease. Doxorubicin has been the most frequently studied agent and is reportedly the most active single drug, with response rates of 10%-46% when used singly or in combination (1-5). However, a recent large series from the Eastern Cooperative Oncology Group reported only seven responses among 51 patients (14% response rate) to doxorubicin alone and two responses among 24 patients to doxorubicin combinations (6). Few other drugs have been tested in adequate prospective trials. A phase II study with vindesine showed minimal activity for this vinca alkaloid (one partial response among 19 evaluable patients) (9). Accumulated reports suggest that alkylating agents probably have modest activity against mesothelioma (1,5).

Although DDP has proven useful in the treatment of multiple malignancies, previous experience in malignant mesothelioma has been relatively limited. Aisner and Wiernik (5) combined scattered case reports and found only two responses among 22 patients with mesothelioma treated with various doses of DDP. Dabouis et al (10) reported a single complete response in nine patients treated with low-dose DDP. Recently, a preliminary report has suggested activity of intracavitary DDP in pleural and peritoneal mesotheliomas (11). The current trial confirms DDP's modest activity, with a response rate of 13% (range, 4%-31%). While it has limited activity as a single agent, there are a few reports of DDP in combination with other drugs (4,5,12). Recently, four of six patients with mesothelioma treated with DDP and doxorubicin in combination were reported to respond (one complete and three partial responses), suggesting a possible improvement in response rate with the combination (12). Because DDP does have modest but possibly useful activity at the dosage and schedule used here, further investigation is warranted to better define the activity of this agent in combination studies.

REFERENCES

1. LEGHA S, and MUGGIA F. Therapeutic approaches to malignant mesothelioma. Cancer Treat Rev 4:13-25, 1977.
2. ANTMAN K, BLUM R, GREENBERGER J, ET AL. Multimodality therapy for malignant mesothelioma based on a study of natural history. Am J Med 68:356-362, 1980.
3. BRENNER J, SORDILLO PD, MAGILL GB, ET AL. Malignant mesothelioma of the pleura; review of 123 patients. Cancer 49:2431-2435, 1982.
4. CHAHINIAN AP, PAJAK TF, HOLLAND JF, ET AL. Diffuse malignant mesothelioma: prospective evaluation of 69 patients. Ann Intern Med 96:746-755, 1982.
5. AISNER J, and WIERNIK PH. Chemotherapy in the treatment of malignant mesothelioma. Semin Oncol 8:335-343, 1981.
6. LERNER H, SCHOENFELD D, MARTIN A, ET AL. Malignant mesothelioma: the Eastern Cooperative Oncology Group experience. Cancer 52:1981-1985, 1983.
7. HAYES DM, CVITKOVIC E, GOLBEY RB, ET AL. High-dose *cis*-platinum diamminedichloride: amelioration of renal toxicity by mannitol diuresis. Cancer 39:1372-1381, 1977.
8. KRIS MG, GRALLA RJ, TYSON LB, ET AL. Improved control of cisplatin-induced emesis with high-dose metoclopramide and with combinations of metoclopramide, dexamethasone and diphenhydramine: results of consecutive trials in 255 patients. Cancer. In press.
9. KELSEN DP, GRALLA R, CHENG E, ET AL. Vindesine in the treatment of malignant mesothelioma: a phase II study. Cancer Treat Rep 67:821-822, 1983.
10. DABOUIS G, LEMEVEL B, and CORROLLER J. Treatment of diffuse pleural malignant mesothelioma by cisdichlorodiammine platinum (C.D.D.P.) in nine patients. Cancer Chemother Pharmacol 5:209-210, 1981.
11. PFEIFLE CE, HOWELL SB, and MARKMAN M. Intracavitary cisplatin for mesothelioma. Proc ASCO 3:258, 1984.
12. ZIDAR BL, PUGH RP, SCHIFER LM, ET AL. Treatment of six cases of mesothelioma with doxorubicin and cisplatin. Cancer 52:1788-1791, 1983.

Phase II Trials of Hexamethylmelamine, Dianhydrogalactitol, Razoxane, and β-2′-Deoxythioguanosine as Single Agents Against Advanced Measurable Tumors of the Pancreas[1,2]

Gastrointestinal Tumor Study Group[2,*]

Phase II trials of several single agents demonstrated only minimal objective response rates in patients with pancreatic carcinoma and measurable tumors: hexamethylmelamine (7%; four responses among 55 patients); dianhydrogalactitol (2.5%; one response among 40 patients); razoxane (7%; two responses among 29 patients); and β-2′-deoxythioguanosine (6%; two responses among 32 patients). Among patients with a good performance status (0–2) and no prior chemotherapy, response rates were 8% for hexamethylmelamine (two responses among 26 patients); 8% for dianhydrogalactitol (one response among 13 patients); 8% for razoxane (one response among 12 patients); and 10% for β-2′-deoxythioguanosine (two responses among 20 patients). None of these agents given by the methods of this study offers substantive benefit to the patient with advanced pancreatic cancer. [Cancer Treat Rep 69:713–716, 1985]

The Gastrointestinal Tumor Study Group has undertaken a series of phase II trials in patients with metastatic and measurable pancreatic cancer. An early set of studies has been published (1).

The trials presented in this paper include the single agents hexamethylmelamine (HXM) (2,3), dianhydrogalactitol (DAG) (4,5), razoxane (ICRF-159) (6,7), and β-2′-deoxythioguanosine (βTGdR). Each agent was studied since it was believed to have a potential role for inclusion in future combination therapy.

MATERIALS AND METHODS

Patients were considered eligible provided they had pathologically confirmed adenocarcinoma of the pancreas. The source of the primary lesion was to be proven on the basis of either surgical inspection or a clearly defined radiographic defect of the pancreas. Tumor was required to be measurable by palpation or x-ray. A liver scan could be employed provided it demonstrated a clearly defined perfusion defect at least 5 cm in greatest diameter. Sonography or computerized tomographic scanning was not accepted as a method for assessing measurable disease. Hepatomegaly was considered a suitable indicator if the liver contained proven metastases and was enlarged at least 5 cm below the xiphoid or midclavicular costal margin. Patients were required to be at least 3 weeks beyond any abdominal surgery involving resection or bypass or 2 weeks beyond an abdominal exploration including patients with biopsy only, 1 month beyond any previous radiation therapy or chemotherapy, and at least 7 weeks beyond any treatment with a nitrosourea.

Patients must have had adequate bone marrow function, including wbc count $\geqslant 4000/\text{mm}^3$ and platelet count $\geqslant 100{,}000/\text{mm}^3$. Patients were ineligible if they had: active infection, acute pancreatitis, prior malignant tumors (other than basal or squamous cell carcino-

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[*] *Reprint requests to:* Gastrointestinal Tumor Study Group, Operations Office, The EMMES Corporation, 11325 Seven Locks Rd, Suite 214, Potomac, MD 20854.

ma), severe anorexia, nausea or frequent vomiting, or caloric intake of < 1200 calories/day. All patients were fully informed regarding risks, limitations, and side effects of the treatment and provided a written informed consent in accordance with institutional and federal guidelines.

Study parameters included standard tests required to assess response and toxicity on a regular basis, with supplemental assessments to quantitate response and toxicity employed as required. Treatment was planned to continue for at least 6 weeks. Patients with disease progression at or after 6 weeks could be assigned to an additional treatment arm. Patients receiving a second protocol treatment are termed cross-over patients and are only included in this report for the evaluation of response. Patients were continued in the study until there was evidence of disease progression or symptomatic deterioration, although patient follow-up continued until death. Criteria for response are as follows: *complete response*—the disappearance of all measurable lesions observed at least 6 weeks after the start of therapy; and *partial response*—a decrease of ⩾ 50% in the product of the largest perpendicular diameters of the most clearly measurable area of malignant disease, with no increase in size of other known areas of malignant disease and no new areas of malignant disease. This result must persist at least 6 weeks after the start of therapy.

If hepatomegaly represents the indicator lesion, there must be a decrease of at least 30% in the sum of the measurements beneath the xiphoid process and both costal margins at the midclavicular line. Disease progression was defined as an increase of ⩾ 25% over original measurements of the product of the two largest perpendicular diameters of the tumor or the appearance of new areas of malignant disease. All responses were subject to joint review by group members. Survival was calculated from the date of initiation of drug treatment.

Treatment

HXM was given at an initial dose of 320 mg/m^2 in four divided oral doses after meals and at bedtime. Doses were rounded off to the nearest 50 mg, and the dosage was administered indefinitely until objective evidence of disease progression was documented. Modification of HXM included a 25% dosage reduction, for a wbc count of 2000-3000/mm^3 or a platelet count of 50,000-75,000/mm^3, or a 50% reduction, for a wbc count < 2000/mm^3 or a platelet count < 50,000/mm^3. If clinically important peripheral neuropathy or any CNS toxicity was produced, HXM was discontinued.

DAG was administered as a rapid iv bolus for 5 consecutive days at a dose of 25 mg/m^2/day. Leukocyte and platelet counts were obtained on Days 1 and 5 of each course of therapy. The same dosage schedule was repeated on Days 36-40 and at 5-week intervals thereafter. A 50% dosage modification was employed for either a wbc count < 1500/mm^3, a platelet count < 50,000/mm^3, or bleeding; a dosage modification of 25% was employed for either a wbc count < 2000/mm^3 or a platelet count < 75,000/mm^3. Dosage escalation was permitted in 20% increments if there was no dose-limiting hematologic toxicity. There was also a provision for a 25% dosage reduction before the initial course of treatment if the patient had received 4000-6000 rad of radiation therapy to the upper abdomen. If there was no toxicity, the next course was given at 100% of the calculated dose.

βTGdR was administered at a dose of 100 mg/m^2 iv daily for 5 days every 3 weeks. Patients who had received 4000-6000 rad of radiotherapy to the upper abdomen were to be treated at a 25% dose reduction (75 mg/m^2 iv × 5) for the first course. If no toxicity occurred (ie, wbc count nadir > 4000/mm^3, hemoglobin > 10 g), the next course was to be given at 100% of the calculated dose (100 mg/m^2 iv × 5). In the event of residual toxicity (wbc count < 4000/mm^3; hemoglobin < 10 g) on the day of scheduled treatment, the interval between the first days of courses was increased to 4 weeks. Dose modification for nadir toxicity was similar to that described for DAG.

ICRF-159 was administered at a dose of 850 mg/m^2/day orally for 3 consecutive days. Each day's dose was divided into three approximately equal portions, administered every 8 hours. Courses of therapy were repeated every 4 weeks. Dose modifications for low wbc and platelet count nadirs included a 25% dose reduction, for a wbc count of 2000-3000/mm^3 or a platelet count of 50,000-75,000/mm^3, and a 50% dose reduction, for a wbc count < 2000/mm^3 or a platelet count < 50,000/mm^3. Patients who had received 4000-6000 rad of radiation therapy to the upper abdomen were treated at a 25% reduction for the first course. If there was no toxicity, the second course was given at 100% of the calculated dose.

These phase II studies were part of a larger set of studies which included treatment arms of maytansine; chlorozotocin; 5-FU, doxorubicin, and mitomycin; streptozocin, mitomycin, and 5-FU; streptozocin, doxorubicin, and semustine; and cisplatin. Eligibility requirements for all arms were not identical; treatment results for these arms will be reported elsewhere.

RESULTS

Table 1 summarizes patient characteristics and response results. Twenty patients have been excluded from the analysis for reasons of eligibility (14) or for refusal to participate after entry (6). Of these 20 patients, four received HXM, eight received DAG, four

TABLE 1.—Patient characteristics and response

Characteristic*	No. of patients	Response		
		No.	Duration (wks)	Survival (wks)
HXM				
Initial treatment, PS 0-2	26	2	9, 8	103, 53
Prior chemotherapy, PS 0-2	17	1	50+	55+
PS 3	1	0		
Cross-over	11	1	10	20
Total	55	4		
DAG				
Initial treatment, PS 0-2	13	1	7	27
Prior chemotherapy, PS 0-2	13	0		
PS 3	9	0		
Cross-over	5	0		
Total	40	1		
ICRF-159				
Initial treatment, PS 0-2	12	1	103	192
Prior chemotherapy, PS 0-2	8	1	114	134
PS 3	5	0		
Cross-over	4	0		
Total	29	2		
βTGdR				
Initial treatment, PS 0-2	20	2	29, 8	65, 53
Prior chemotherapy, PS 0-2	4	0		
PS 3	7	0		
Cross-over	1	0		
Total	32	2		

* PS = performance status.

received ICRF-159, and four received βTGdR. Ten of the ineligible patients had inadequate lesions for measurement, while four had tumors of other ineligible pathologic types. Responses have been observed on each arm. One response each on the HXM and on the ICRF-159 arms was complete, while all the remaining responses were partial. HXM and ICRF-159 were observed to produce responses in previously treated patients. There was no clear relationship between PS and response. Four of the eight non-cross-over responses were in patients with a PS of 2. Patients with a PS of 2 represent 51% of the study subjects.

Median survival times for first-line protocol therapy are 10.9, 9.3, 9.7, and 9.8 weeks for HXM, DAG, ICRF-159, and βTGdR, respectively.

Table 2 shows some of the observed toxic effects. Hematologic toxic effects were most severe with ICRF-159 and βTGdR treatment, although both were in the acceptable range. Vomiting was most common with HXM treatment. Other problems included three in-

TABLE 2.—Toxic effects associated with treatment*

Toxic effect	% with grade of toxicity			
	HXM (n = 44)	DAG (n = 35)	ICRF-159 (n = 25)	βTGdR (n = 31)
Wbc count nadir (mm^3)				
2000-4500	20	29	52	45
1000-1999	5	3	20	13
< 1000	0	3	12	10
Platelet count nadir (mm^3)				
50,000-129,999	14	17	20	26
25,000-49,999	5	9	8	6
< 25,000	2	0	4	13
Severe vomiting	20	0	4	3

* Excludes cross-over.

stances of reversible neurologic disturbances (vertigo, delusions, and visual disturbances) in patients treated with HXM. No treatment-related deaths occurred.

DISCUSSION

The single agents employed in this study demonstrated only minimal activity. This was the case despite efforts to ensure treatment of at least 12 ideal patients with each drug. These agents do not warrant further study in pancreatic cancer as single agents by the methods we employed. The observation that responders, even after prior therapy, enjoyed a substantially better survival suggests that additional phase II chemotherapy trials are necessary to find more active agents for patients with pancreatic carcinoma.

REFERENCES

1. Schein PS, Lavin PT, Moertel CG, et al. GITSG: randomized phase II clinical trial of adriamycin, methotrexate and actinomycin-D in advanced measurable pancreatic carcinoma, a GITSG report. Cancer 42:19–22, 1978.
2. Wilson WL, Bisel HF, Cole D, et al. Prolonged low dosage administration of hexamethylmelamine (NSC-13785). Cancer 25: 268–270, 1970.
3. Bruckner HW, Storch JA, Brown JC, et al. Phase II trial of combination chemotherapy for pancreatic cancer with 5-fluorouracil, mitomycin-C, and hexamethylmelamine. Oncology 40:161–164, 1983.
4. Elson LA, Varman M, Ross WJ, et al. Toxicity, hematological effects and anti-tumor activity of epoxides derived from disubstituted hexitols mode of action of mannitol, myleran and dibromomannitol. Eur J Cancer 4:617–620, 1968.
5. Németh L, Institóris L, Somfai S, et al. Pharmacologic and antitumor effects of 1,2:5,6-dianhydrogalactitol (NSC-132313). Cancer Chemother Rep 56:593–602, 1972.
6. LeServe AW, and Hellmann K. Metastases and the normalization of tumor blood vessels by ICRF-159: a new type of drug action. Br Med J 1:597–601, 1972.
7. Clinical Brochure. ICRF-159 (NSC-168780). National Cancer Institute, June 1978.

Subrenal Capsule Assay: Feasibility of Transporting Tissues to a Central Facility for Testing[1,2]

Donald E. Slagel,[3,*] Philip DeSimone,[4] Marcus Dillon,[3] Doreen J. LePage,[5] and Arthur E. Bogden[5]

A subrenal capsule assay has been developed by Bogden et al (1,2) and utilized with some success for evaluating the effectiveness of chemotherapeutic drugs against the individual patient's tumor (3,4).

Because of the sophisticated nature of the subrenal capsule assay and the need for reproducibility, we thought that it would be important to determine if the surgical biopsy could be routinely submitted from a remote site and be tested at a central facility. We report here the results of submitting 50 tumor specimens obtained at the University of Kentucky Albert B. Chandler Medical Center to Mason Research Institute for testing.

MATERIALS AND METHODS

Surgical biopsies were taken using sterile procedure and placed in a Petri dish. Tumor was trimmed of connective tissue and necrotic and hemorrhagic areas and was sliced in long pieces about 1-2 mm thick. These were immersed in McCoy's medium containing 10% newborn calf serum and 1% penicillin-streptomycin at room temperature. Vials were completely filled with the medium, placed in a styrofoam container, and shipped at ambient temperature via overnight delivery service. The remainder of this protocol was carried out at Mason Research Institute, as previously described (3).

Briefly, normal immunocompetent CDF_1 (BALB/c × DBA/2) mice are used as xenograph recipients. The tumor fragment measuring 10 × 10 × 10 ocular micrometer units (1 mm^3) is cut and inserted in a small slit made in the mouse exteriorized kidney capsule. The implant fragment is measured in situ under a dissecting scope. On Day 6 the mice are killed and the tumor is again measured in situ. For an assay to be considered evaluable the change in the tumor size must be greater than or equal to -0.5 ocular micrometer units.

RESULTS

Surgical biopsies, either of the primary tumor or tumor cell-containing lymph node, from 73 patients have been evaluated during a recent 32-month period. A variety of tumor histologic types were represented: 27% were squamous cell carcinoma; 25% were adenocarcinoma; and 18% were small cell carcinoma of the lung. Because of day-of-the-week differences in performing surgery, the transplanted tumor specimens could be categorized into three groups with 1, 2, and 3 days intervening between surgery and the initiation of the subrenal capsule assay. The evaluable assay rates for these three groups are summarized in table 1. There was a high rate of evaluable specimens when the surgical specimen was assayed 1, 2, or 3 days following surgery (83%, 91%, and 81%, respectively). We saw no relationship between the evaluable assay rate and whether the surgical biopsy came from primary tumor tissue or tumor cell-infiltrated lymph node.

We have also determined the evaluable assay rate at 4-month intervals during the 14-month period. A breaking-in period during which communication and shipping procedures were being optimized was observed. Table 1 shows the evaluable assay going from 70% during the first half of the collection period to 90% during the last half.

These results illustrate that it is feasible to carry out the subrenal capsule assay on surgical specimens submitted from a remote site to a central facility. This allows clinical evaluation of the subrenal capsule assay using a large number of patients with different histologic tumor

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[2] Supported in part by contract N01-CM-07325 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[3] Department of Surgery, University of Kentucky Medical Center, Lexington.

[4] Department of Medicine, Veterans Administration Hospital, Lexington, KY.

[5] Laboratory of Immunobiology and Experimental Oncology, EG&G Mason Research Institute, Worcester, MA.

[*] *Reprint requests to:* Donald E. Slagel, PhD, Department of Surgery, Division of Neurosurgery, University of Kentucky Medical Center, Lexington, KY 40536-0084.

types which are gathered from a wide geographic region. Thus, the inclusion of the subrenal capsule assay in treatment protocols carried out by regional cancer study groups becomes practical. If the use of the subrenal capsule assay becomes a clinically useful predictor of chemotherapeutic compound response, this work suggests that regional cancer centers can carry out the assay for chemotherapeutic treatment of patients in surrounding local communities.

TABLE 1.—Evaluable assay rate of transported tumor specimens

	Evaluable assay fractions (%)*
Days intervening between surgery and testing	
1	24/29 (83)
2	21/23 (91)
3	9/11 (82)
4	5/7 (71)
Initial period during optimization of the procedure†	
1st 4 mos	12/16 (75)
2nd 4 mos	10/13 (77)
3rd 4 mos	10/12 (83)
4th 20 mos	29/32 (91)
Evaluable assay for different tumor types	
Squamous cell carcinoma	20/20 (100)
Small cell carcinoma	10/13 (77)
Adenocarcinoma	14/18 (78)

* Values = No. of evaluable specimens/Total No. Evaluable assay = control change in tumor size $\geqslant -0.5$ ocular micrometer units.

†Total = 61/73 (82%).

REFERENCES

1. BOGDEN AE, KELTON DE, COBB WR, ET AL. A rapid screening method for testing chemotherapeutic agents against human xenografts. *In* Proceedings of the Symposium on the Use of Athymic (Nude) Mice in Cancer Research (Houchens DP, and Ovejera AA, eds). New York, Gustav Fischer, Inc, 1978, pp 231-250.
2. BOGDEN AE, HASKELL PM, LEPAGE DJ, ET AL. Growth of human tumor xenografts implanted under the renal capsule of normal immunocompetent mice. Exp Cell Biol 47:281-293, 1979.
3. BOGDEN AE, COBB WR, LEPAGE DJ, ET AL. Chemotherapy responsiveness of human tumors as first transplant generation xenografts in the normal mouse: six-day subrenal capsule assay. Cancer 48:10-20, 1981.
4. GRIFFIN TW, BOGDEN AE, REICH SD, ET AL. Initial clinical trials of the subrenal capsule assay as a predictor of tumor response of chemotherapy. Cancer 52:2185-2192, 1981.

Treatment of Refractory Hodgkin's Disease With Teniposide, Cisplatin, Hexamethylmelamine, and Prednisolone: Cancer and Leukemia Group B Trial 8171[1,2]

Daniel R. Budman,[3,*] James Anderson,[4] J. Paul Obrecht,[5] George P. Canellos,[6] Patrick Creaven,[7] Larry Norton,[8] and Arlen Gottlieb[9]

The treatment of advanced Hodgkin's disease has markedly improved with the advent of combination chemotherapy (1,2). However, a substantial number of patients fail to attain a complete remission, and one-half of the patients who achieve a complete remission with chemotherapy eventually relapse (3). In an attempt to design a non-cross-resistant, noncardiotoxic salvage regimen, the Cancer and Leukemia Group B (CALGB) undertook a pilot trial with teniposide, cisplatin, hexamethylmelamine, and prednisolone, since these agents had previously shown activity in this disease and were synergistic in animal model systems.

MATERIALS AND METHODS

Patients with histologically documented Hodgkin's disease who had failed one to three multiple-drug treatment programs, had a performance status $\leqslant 2$ (ambulatory), and had anticipated survival > 3 months were eligible for the study. Central pathologic review was required. All toxic effects of prior therapy had to be resolved before entry into the study.

Laboratory values included initial wbc count $\geqslant 3500/\mu l$, platelet count $\geqslant 100{,}000/\mu l$, bilirubin < 2.0 mg/dl, and serum creatinine $\leqslant 1.5$ mg/dl. All patients had measurable disease. Informed consent was obtained.

Initial drug dosages were: teniposide, 60 mg/m^2 iv on Day 1; cisplatin, 40 mg/m^2 iv on Day 1; hexamethylmelamine, 100 mg/m^2 orally on Days 2-8; and prednisolone, 32 mg/m^2 (or an equivalent corticosteroid) orally on Days 2-8. Treatment was repeated every 21 days. In the absence of hematologic toxicity on Days 8 and 15, the next cycle of teniposide was escalated to a dose of 80 mg/m^2. All patients were followed weekly for a minimum of 4 weeks. Responses and toxicity were scored as previously described (2).

RESULTS

Fifty-six patients were entered in the study. Seven patients were judged to be ineligible: two with incomplete data, two with inadequate laboratory parameters, two with inadequate performance status, and one with non-Hodgkin's pathology on review. Seventy-eight percent of study patients were < 40 years of age, with a slight male predominance (table 1). Ninety-four percent were stage III or IV, with B symptoms in 38 patients. Patients had received an average of eight drugs, with 47 of the 49 evaluable patients receiving two to three combinations. During this study, one-half of the patients had the iv teniposide dosage escalated for their second cycle. Ninety-six percent received their drugs on schedule.

Major responses (complete plus partial) were seen in 31% of the patients, usually occurring after the first cycle of therapy, with a mean duration of 4.8 months. Response did not correlate with stage of disease, and all responders eventually relapsed. Approximately 20% of the patients exhibited significant toxicity (grade III-IV). Three of 15 responding patients were removed from the study because of toxic effects.

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[3] Don Monti Division of Oncology, Department of Medicine, North Shore University Hospital, and Department of Medicine, Cornell University Medical College, Manhasset, NY.

[4] Cancer and Leukemia Group B, Brookline, MA.

[5] Division of Oncology, Kantonsspital, Basel, Switzerland.

[6] Division of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA.

[7] Department of Medical Oncology, Roswell Park Memorial Institute, Buffalo, NY.

[8] Department of Neoplastic Disease, Mount Sinai Medical Center, New York, NY.

[9] Division of Hematology/Oncology, Upstate Medical Center, Syracuse, NY.

[*] *Reprint requests to:* Daniel R. Budman, MD, Department of Medicine, North Shore University Hospital, 300 Community Dr, Manhasset, NY 11030.

TABLE 1.—Patient characteristics, response, and toxicity

Characteristic	No. of patients
Total entered	56
Total evaluable	49
Age distribution (yrs)	
< 40	38
40-59	7
60+	4
Sex	
Male	29
Female	20
Stage at entry	
II	3
III	4
IV	42
Mean performance status (range): 1 (0-2)	
Histology	
Nodular sclerosis	33
Mixed cellularity	13
Lymphocyte depleted	2
Unclassifiable	1
Prior therapy	
Radiation	31
Chemotherapy	49
⩽ 4 drugs	2
5-8 drugs	32
⩾ 9 drugs	15
Response	
Complete	3 (6%)
Partial	12 (25%)
Mean duration of response in mos (range): 4.8 (1.9-11.0)	
Toxicity (grade III-IV)	
Leukopenia	12%
Thrombocytopenia	14%
Infection	18%
Allergy	2%
Nausea/vomiting	18%

DISCUSSION

This drug combination was chosen because of preclinical synergy between cisplatin and the epipodophyllotoxins (4), synergy in treating several human tumors, and previous phase II activity in Hodgkin's disease (5-9). The scheduling and dosage was designed for outpatient treatment.

Although a major response rate of 31% (95% confidence limits, 18%-44%) was obtained in this patient population, this response rate differs little from that obtained with active phase II agents (5-7). In addition, use of this combination did not result in long-term disease-free survival in responding patients. Hence, this combination does not offer any advantage as salvage therapy. Prior chemotherapy may have minimized the maximum potential response rate of this combination chemotherapy as is seen when ABVD (doxorubicin, bleomycin, vinblastine, and dacarbazine) is used as salvage therapy, compared to its efficacy in previously untreated patients (10). Clinical evaluation of new combinations of agents found to be active in Hodgkin's disease may require their use as primary therapy to assess their true response rate and durability.

REFERENCES

1. DEVITA VT, CANELLOS GP, and MOXLEY JH, III. A decade of combination chemotherapy of advanced Hodgkin's disease. Cancer 30:1495-1504, 1972.
2. COOPER MR, PAJAK TF, NISSEN NI, ET AL. A new effective four drug combination of CCNU (1-2-chlorethyl 1-3-cyclohexyl-1-nitrosourea) (NSC 79038), vinblastine, prednisone, and procarbazine for the treatment of advanced Hodgkin's disease. Cancer 46:654-662, 1980.
3. KAPLAN HS. Hodgkin's disease: unfolding concepts concerning its nature, management and prognosis. Cancer 45:2439-2474, 1980.
4. SCHABEL FM, JR, TRADER MW, LASTER WR, JR, ET AL. *cis*-Dichlorodiammineplatinum(II): combination chemotherapy and cross-resistance studies with tumors of mice. Cancer Treat Rep 63:1459-1473, 1979.
5. CORDER MP, ELLIOT TE, MAGUIRE LC, ET AL. Phase II study of *cis*-dichlorodiammineplatinum(II) in stage IVB Hodgkin's disease. Cancer Treat Rep 63:763-766, 1979.
6. OMURA GA, BROUN GO, PAPPS J, ET AL. Phase II study of hexamethylmelamine in refractory Hodgkin's disease, other lymphomas, and chronic lymphocytic leukemia. Cancer Treat Rep 65:1027-1029, 1981.
7. MACBETH FR. VM 26: phase I and II studies. Cancer Chemother Pharmacol 7:87-91, 1982.
8. ISSELL BF. The podophyllotoxin derivatives: VP 16-213 and VM 26. Cancer Chemother Pharmacol 7:73-80, 1982.
9. BRITISH NATIONAL LYMPHOMA INVESTIGATION. Value of prednisone in combination chemotherapy of stage IV Hodgkin's disease. Br Med J 3:413-414, 1975.
10. BONADONNA G, SANTORO A, ZUCALI R, ET AL. Improved 5-year survival in advanced Hodgkin's disease by combined modality approach. Cancer Clin Trials 2:217-226, 1979.

Interferon and Doxorubicin in Renal Cell Carcinoma[1,2,3]

Hyman B. Muss,* Charles Welander, Mary Caponera, Ken Reavis, Julia M. Cruz, M. Robert Cooper, Don V. Jackson, Jr, Frederick Richards, II, John J. Stuart, Charles L. Spurr, Douglas R. White, Patricia J. Zekan, and Robert L. Capizzi[4]

Interferons have demonstrable antitumor activity (1) and have resulted in tumor regression in patients with metastatic renal cell carcinoma. In addition, recent studies have demonstrated in vitro synergy between interferon and cytotoxic agents in a variety of solid tumor types (2-5). Recombinant interferon α_2 (rIFN-α_2, Schering Corp, Bloomfield, NJ) was given by iv and sc routes simultaneously to achieve rapid (iv) but sustained (sc) plasma levels. Our laboratory data showed marked in vitro synergism when certain types of tumor cells had 1 hour of pre-exposure to rIFN-α_2 followed by doxorubicin at an in vitro concentration $^1/_{100}$ of an achievable peak plasma level, following an iv dose of 50 mg/m^2 (0.006 μg/ml). Pharmacologic data obtained in our phase I trials of rIFN-α_2 indicated that the dose and schedule used in this study would maximize rIFN-α_2 cell exposure during

TABLE 1.—Patient characteristics

Patient No.	Age (yrs)/ PS*	Disease-free interval (mos)	Metastatic sites	Nephrectomy	Prior treatment†	No. of courses	Response	Comments
1	36/1	0	Lung	Yes	None	10	Stable	Off treatment, failure
2	54/2	0	Liver	Yes	M	8	Stable	Off treatment, fever
3	75/1	1	Lung	Yes	None	5	Progressed	—
4	48/1	31	Lung	Yes	M-L-V-C	3	Progressed	—
5	71/1	0	Lung	Yes	IFN	5	Stable	Off treatment, failure
6	59/2	2	Bone	No	None	14	Progressed	Progressive disease after 3 courses, then stable
7	60/1	0	Lung, skin, liver	No	None	5	Stable	Off treatment, failure
8	51/1	12	Lung	Yes	P-L-V-C, M	3	Progressed	
9	65/1	0	Lung	No	None	2	Not evaluable	Abnormal multigated cardiac scan
10	64/2	0	Lung, nodes, brain‡	No	None	3	Progressed	
11	62/2	0	Lung, soft tissue, renal bed	Yes	None	4	Stable	Off treatment, failure
12	44/1	0	Lung, adrenal	No	M-L-V-C	3	Not evaluable	Refused treatment
13	60/2	0	Lung, liver	No	None	5	Stable	Off treatment, failure
14	51/2	0	Bone	No	None	5	Stable	—
15	64/3	10	Adrenal, abdominal metastasis	Yes	None	2	Progressed	Off treatment, failure
16	46/1	0	Lung	No	None	3	Progressed	Off treatment, failure
17	33/1	0	Renal bed, lung	No	None	3§	Stable	—

* PS = performance status, Zubrod scale.

†M = megestrol, L = lomustine, V = vinblastine, C = cyclophosphamide, IFN = interferon, and P = medroxyprogesterone.

‡Radiation therapy.

§After 3 courses.

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[3] Recombinant interferon α_2 was supplied by the Schering Corporation, Bloomfield, NJ.

[4] Oncology Research Center and Departments of Medicine and Obstetrics and Gynecology, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC.

* *Reprint requests to:* Hyman B. Muss, MD, Department of Medicine, Section on Hematology/Oncology, Bowman Gray School of Medicine, Wake Forest University, 300 S Hawthorne Rd, Winston-Salem, NC 27103.

the short doxorubicin infusion. These preclinical data were used as the basis of a phase II study of rIFN-α_2 and doxorubicin in renal cell carcinoma.

METHODS AND RESULTS

Seventeen patients with adequate hematologic, renal, and liver functions, measurable or evaluable lesions, a performance status of 0-2 (Zubrod scale), and appropriate informed consent were treated. No patient had received prior doxorubicin.

Treatment consisted of oral acetaminophen 60 minutes before interferon therapy; 10×10^6 IU/m^2 of rIFN-α_2 was given sc followed immediately by a 30-minute iv infusion of identical dosage. Thirty minutes after completion of the rIFN-α_2 infusion, doxorubicin (20 mg/m^2 iv) was given over 2 hours. Patients were given three courses of this therapy at 1-week intervals. Those with stable or responding disease were continued on the same regimen at 2-week intervals until the doxorubicin dose reached 550 mg/m^2. Hematologic toxicity required dose reduction for doxorubicin, but not for rIFN-α_2.

Toxicity consisted of fever or flu-like symptoms in 16 of 17 patients; nausea and vomiting in ten; transient elevations of liver function tests in three; and chills, stomatitis, and alopecia in 12, three, and two, respectively. After 81 courses, a wbc count $< 2000/\mu l$ was noted in only one patient (1500 and 1800/μl on two occasions), a platelet count of $< 100{,}000/\mu l$ in another (64,000, 92,000, and 92,000/μl on three occasions), and a hemoglobin < 9 g/100 ml in two others. There were no responses in 15 evaluable patients (95% confidence interval of 0%-18%), including 12 who had had no prior therapy (95% confidence interval of 0%-22%) (table 1).

CONCLUSIONS

The present regimen, although ineffective in renal cell carcinoma, was well tolerated and no unanticipated toxicity was observed. Our laboratory is currently exploring other schedules and combinations of rIFN-α_2 and cytotoxic agents for use in phase I and II trials.

REFERENCES

1. KIRKWOOD JM, and ERNSTOFF MS. Interferons in the treatment of human cancer. J Clin Oncol 2:336-352, 1984.
2. BALKWILL FR, and MOODIE EM. Positive interactions between human interferon and cyclophosphamide or adriamycin in a human tumor model system. Cancer Res 44:904-908, 1984.
3. AAPRO MS, ALBERTS DS, and SALMON SE. Interaction of human leukocyte interferon with vinca alkaloids and other chemotherapeutic agents against human tumors in clonogenic assay. Cancer Chemother Pharmacol 10:161-166, 1983.
4. YAMAMOTO S, TANAKA H, KANAMORI T, ET AL. In vitro studies on potentiation of cytotoxic effects of anticancer drugs by interferon on a human neoplastic cell line (HeLa). Cancer Lett 20:131-138, 1983.
5. WELANDER C, GAINES JT, HOMESLEY HD, ET AL. In vitro synergistic effects of recombinant human interferon alpha$_2$ (rIFN-α_2) and doxorubicin on human tumor cell lines. Proc ASCO 2:42, 1983.

Phase II Study of Mitolactol in Advanced Malignant Melanoma[1,2]

Nevin Murray,* Hulbert Silver, Amil Shah, and Kenneth Wilson[3,4]

Metastatic malignant melanoma is notoriously resistant to chemotherapy, particularly after previous drug treatment. Mitolactol has been reported to produce response rates up to 30% (1,2). One of these studies reported responses to mitolactol in patients previously treated with dacarbazine and a nitrosourea (2). Subsequent trials of either mitolactol alone, using slightly differing doses and schedules (3,4), or mitolactol and semustine (5) did not achieve such encouraging results. We report our experience with an intermittent oral regimen for previously treated patients with advanced malignant melanoma.

All patients had biopsy-proven metastatic melanoma with measurable disease in an unirradiated area. Mitolactol was offered as a second-line chemotherapy when dacarbazine or a nitrosourea had failed. Mitolactol, at a dose of 180/mg/m^2/day × 10 days, was given orally at 28-day intervals. The dose was escalated by 10% in subsequent cycles in the absence of significant side effects.

The characteristics of 21 patients with metastatic melanoma treated on this study are shown in table 1. Nineteen patients developed progressive disease within 2 months. Two patients, one with lung metastasis and another with subcutaneous lesions, had stable disease. However, a careful review of the pretreatment growth rate of these indolent melanomas indicated no change in the doubling time related to drug treatment.

The median survival time of the entire group was 5 months. The median survival time for patients with progressive disease was 4 months; for stable disease patients, it was 14 months. The long survival of the two stable disease patients was probably due to their slow-growing tumor rather than a treatment benefit.

TABLE 1.—Patient characteristics

Characteristic	No. of Patients (%)
Evaluable	21
Median age in yrs (range): 57 (34–70)	
Sex	
Male	8
Female	13
Performance status*	
0–1	15 (71)
2–3	6 (29)
Sites of disease	
Soft tissue	8 (38)
Lung	7 (34)
Liver	3 (14)
Abdomen (excluding liver)	3 (14)
Prior chemotherapy	
Dacarbazine	18 (85)
Lomustine	2 (10)
None	1 (5)
Prior radiotherapy	5
No. of cycles of mitolactol	
1–2	19
4–10	2
Toxicity	
Nausea and vomiting	3 (14)
Leukopenia (wbc count $< 3000/\text{mm}^3$)	7 (33)
Thrombocytopenia (platelet count $< 100{,}000/\text{mm}^3$)	4 (19)
Infection	0
Bleeding	0
Response rates	
Partial response ($>$ 50% tumor area reduction)	0
Stable disease ($<$ 50% tumor area reduction)	2
Disease progression ($>$ 25% tumor area increase)	19

* Eastern Cooperative Oncology Group criteria.

In conclusion, although mitolactol can be given safely with this dose schedule, we found the response rate of the drug in patients with previously treated metastatic melanoma to be $< 15\%$ (rejection error $< 5\%$). This may be due to cross-resistance with other alkylating agents or it may merely indicate lack of activity of this drug. Further studies of this schedule of mitolactol for patients previ-

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[2] Mitolactol was supplied by the National Cancer Institute, Bethesda, MD.

[3] Melanoma Tumour Group, Cancer Control Agency of British Columbia, and Department of Medicine, University of British Columbia, Vancouver, Canada.

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* *Reprint requests to:* Dr. N. Murray, Cancer Control Agency of British Columbia, 600 W 10th Ave, Vancouver, British Columbia, V5Z 4E6, Canada.

ously treated with chemotherapy for advanced malignant melanoma are not recommended.

REFERENCES

1. ANDREWS NC, WEISS AJ, WILSON W, ET AL. Phase II study of dibromodulcitol (NSC-104800). Cancer Chemother Rep 58:653-660, 1974.
2. BELLET RE, CATALANO RB, MASTRANGELO MJ, ET AL. Positive phase II trial of dibromodulcitol in patients with metastatic melanoma refractory to DTIC and a nitrosourea. Cancer Treat Rep 62:2095-2099, 1978.
3. MEDINA W, and KIRKWOOD JM. Phase II trial of mitolactol in patients with metastatic melanoma. Cancer Treat Rep 66:195-196, 1982.
4. HOPKINS J, RICHARDS F, CASE D, ET AL. A phase II study of dibromodulcitol in stage IV melanoma. Proc ASCO 1:696, 1982.
5. CREAGAN ET, AHMANN DL, SCHUTT AJ, ET AL. Phase II study of mitolactol and semustine combination chemotherapy for advanced malignant melanoma. Cancer Treat Rep 66:1425-1426, 1982.

Phase II Study of Aclarubicin in Previously Untreated Patients With Advanced Soft Tissue Sarcoma: A Southeastern Cancer Study Group Trial[1,2]

Marcelle Bertrand,[3] Phyllis Multhauf,[3,*] Alfred Bartolucci,[4] Donna Ellison,[4] and Jon Gockerman[5,6]

Aclarubicin, an anthracycline antibiotic isolated from a culture broth of *Streptomyces galilaeus*, has demonstrated therapeutic activity in animal tumor models. Initial phase I data suggested that aclarubicin had fewer side effects than doxorubicin (1-3). Because of the discouragingly low response rate for doxorubicin in the treatment of soft tissue sarcoma (4), this phase II trial was undertaken to investigate the activity of aclarubicin in patients with advanced soft tissue sarcoma previously unexposed to chemotherapy.

Aclarubicin was administered as an iv bolus injection at a dose of 100 mg/m^2 (good marrow reserve) or 75 mg/m^2 (poor marrow reserve, defined as extensive bone metastases, extensive prior radiation therapy, or age > 70 years). Courses were repeated every 3 weeks subject to hematologic recovery. An adequate trial was defined as a minimum of two courses. Treatment was administered to a total dose of 2250 mg/m^2 or until definitive evidence of disease progression.

Patient characteristics and response to treatment are shown in tables 1 and 2. All patients had normal hematologic, hepatic, and renal functions. Patients with cardiac problems precluding anthracycline therapy were not eligible. Of 27 cases entered, four were inevaluable: one case had inadequate data to assess efficacy due to patient noncompliance; upon review of submitted pathologic material, two had inadequate specimens to confirm diagnosis; and one was found to be mesothelioma.

Toxicity was essentially hematologic and gastrointestinal. Of the 19 patients fully evaluable for toxicity, four had severe (500 to < 1000 cells/μl) granulopenia and six had maximal (< 500 cells/μl) granulopenia on the initial course. Seven of these patients had a 50% dose reduction on the second course, per protocol, with no severe granulopenia observed; three were treated at full dose at the

TABLE 1.—Patient characteristics

Characteristic	No. of patients
Median age in yrs (range): 59 (32-75)	
Median Karnofsky performance status (range): 90 (60-100)	
Sex	
Male	13
Female	10
Previous radiation therapy	10
Primary site	
Upper limb	3
Lower limb	7
Retroperitoneum	4
Gastrointestinal	3
Miscellaneous*	6
Cell type	
Malignant fibrous histiocytoma	10
Synovial cell sarcoma	2
Epithelial sarcoma	2
Leiomyosarcoma	2
Miscellaneous†	7
Site(s) of metastasis	
Lung/pleura	16
Liver	7
Abdomen (includes retroperitoneum)	7
Bone	4
Soft tissue (includes skin, lymph nodes)	2
Mediastinum	1

*One patient each: head/neck; breast; uterus; omentum; trunk, unspecified; and unknown primary.

†One patient each: angiosarcoma; undifferentiated sarcoma; malignant schwannoma; liposarcoma; rhabdomyosarcoma; myoblastoma; and leiomyoblastoma.

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[3] City of Hope National Medical Center, Duarte, CA.

[4] Southeastern Cancer Study Group Statistical Center, University of Alabama, Birmingham.

[5] Duke University, Durham, NC.

[6] We are grateful to all of the Southeastern Cancer Study Group investigators who contributed to this study and to the patients who cooperated in this trial. We thank Sunny Ilagan for her excellent secretarial assistance in preparing the manuscript.

[*] *Reprint requests to:* Phyllis Multhauf, Department of Biostatistics, City of Hope National Medical Center, 1500 E Duarte Rd, Duarte, CA 91010.

TABLE 2.—Evaluation of response

Response	No. of patients	Duration in wks*
Partial response (≥ 50% decrease)	1	17.1†
Stable disease (< 50% decrease, < 25% increase)	5	11, 11, 30, 30, 39+
Disease progression (≥ 25% increase)	17	

* Calculated from Day 1 of treatment.
†Time to response, 7.1 wks.

discretion of the investigator. Six patients with stable or better disease received more than two courses of therapy: one remained at a 50% dose after the initial course; two had dose escalations at 25% increments, as tolerated, to a maximum of 100 mg/m^2; and three remained at full dose throughout. Nadir granulocyte counts appeared 2-4 weeks posttherapy, with recovery in 1 week. Cumulative hematologic toxicity could not be assessed. No drug-related death was reported. Transient moderate thrombopenia (60,000 cells/μl) was observed in one patient. Other reported toxic effects included moderate nausea and vomiting (14 patients), mild to moderate hair loss (three), and mild irritation at the site of iv injection (two). ECGs were not consistently performed; however, sinus tachycardia of unclear etiology was observed in one patient after two cycles of therapy. Another patient experienced occasional premature atrial and ventricular contractions associated with transient chest pains. Cardiac ischemic disease was ruled out; the chest pains were felt to be disease-related.

In summary, for the dose and schedule used in this study, we can exclude meaningful activity of aclarubicin in patients with advanced soft tissue sarcoma. Toxicity was characterized by transient, uncomplicated granulopenia, nausea, and vomiting.

REFERENCES

1. Clinical Brochure. Aclacinomycin A (NSC-208734). Bethesda, MD, Investigational Drug Branch, National Cancer Institute, July 1979.
2. OKI T. New anthracycline antibiotics. Jpn J Antibiot 30:70-84, 1977.
3. HORI S, SHIRAI M, HIRANO S, ET AL. Antitumor activity of new anthracycline antibiotics aclacinomycin A, and its analogs and their toxicity. Gann 68:685-690, 1977.
4. SCHOENFELD DA, ROSENBAUM C, HORTON J, ET AL. A comparison of adriamycin versus vincristine and adriamycin and cyclophosphamide versus vincristine, actinomycin D and cyclophosphamide for advanced sarcoma. Cancer 50:2757-2762, 1982.

Phase II Evaluation of Bisantrene in Patients With Advanced Renal Cell Carcinoma[1,2]

W. K. Evans,* F. A. Shepherd, M. E. Blackstein, D. Osoba, and D. Taylor[3]

Bisantrene hydrochloride has antitumor activity against a variety of rodent tumors (1). In the human tumor cloning system, bisantrene demonstrated activity against renal cell carcinoma (2). This phase II study was designed to determine the therapeutic efficacy and toxicity of bisantrene given iv on a weekly × 3 schedule.

PATIENTS AND METHODS

Patients with measurable, histologically documented metastatic renal cell carcinoma were eligible for study provided they had a life expectancy > 12 weeks, an Eastern Cooperative Oncology Group (ECOG) performance status $\leqslant 2$, and gave informed consent. All patients had adequate cardiac, hepatic, and renal functions. Bisantrene (180 mg/m^2) was given weekly for 3 consecutive weeks every 42 days. The drug was diluted in 5% dextrose and water to a concentration of 0.1-0.5 mg/ml and infused through a peripheral vein over 2 hours under protection from light. During the course of the study, various measures, including iv corticosteroid administration, oral nonsteroidal anti-inflammatory medications, and pre- and post-chemotherapy intralipid administration, were attempted in an effort to reduce the incidence of drug-induced phlebitis. The dose was modified according to treatment day blood counts, and was escalated in patients who maintained a wbc count $> 3.5 \times 10^9$/L or a platelet count $> 150 \times 10^9$/L during the first cycle of weekly treatments. Blood pressure was monitored at 15-30-minute intervals during infusions.

Standard response criteria for complete and partial responses were used. A mixed response was defined as a $> 50\%$ decrease in the area of some lesions with a $< 25\%$ increase in the size of other lesions. Stable disease was a $< 25\%$ change in the area for > 3 months.

Treatment characteristics of the 24 patients in this study are summarized in table 1.

TABLE 1.—Patient characteristics

Characteristic	No. of patients
Total	24
Sex	
Male	17
Female	7
Median age in yrs (range): 58 (43-79)	
ECOG performance status	
0	2
1	9
2	13
Sites of tumor involvement	
Lung	20
Bone	10
Liver	5
Nodal	4
Renal bed	3
Brain	2
Thyroid, pancreas, adrenal, tongue	1 (each)
Prior therapy	
Medroxyprogesterone	7
Vinblastine	5
Courses of bisantrene	
1	20
2	11
3	4
4	1
5	1

RESULTS

Four patients were inevaluable for response due to early death from pneumonia (one patient), rapidly progressive hepatic metastases (one), acute lymphoblastic leukemia (one), and thrombocytopenia due to marrow infiltration (one).

There were 20 patients evaluable for response. One patient had a partial response of pulmonary metastases for 47 weeks and survived 75 weeks. Two patients had mixed responses characterized by a $> 50\%$ decrease in the area

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[2] Supported by a grant from the Medical Research Division, American Cyanamid Co, Lederle Laboratories, Pearl River, NY.

[3] Departments of Medicine of the Toronto General Hospital, Mount Sinai Hospital, and the Toronto Bayview Cancer Clinic, Ontario, Canada.

* *Reprint requests to:* Dr. W. K. Evans, Toronto General Hospital, 101 College St, ML 1-052, Toronto, Ontario, Canada M5G 1L7.

of metastatic lesions in the lung of 12 and 15 weeks' duration, but had disease progression in the bone. The survival of these patients was 22 and 40 weeks. There were 16 nonresponding patients; four of these were categorized as stable and survived 67, 68, 76, and 84 weeks. The remaining patients demonstrated tumor progression while on therapy.

Toxicity

Myelosuppression was modest, with a mean nadir granulocyte count of 2.6×10^9/L (range, 0.36–6.3 $\times 10^9$/L). Thrombocytopenia $< 100 \times 10^9$ cells/L was not observed. Gastrointestinal toxicity included mild nausea (six patients) and diarrhea (one). Four patients had transient febrile episodes within 24 hours of drug administration and one developed a maculopapular rash. Two patients had transient hypotension during drug infusion and one developed flushing, tachycardia, hypertension, and bronchospasm during a second bisantrene infusion.

Phlebitis occurred in 18 patients, despite the measures described above. In three cases, recurrent phlebitis resulted in loss of peripheral venous access.

DISCUSSION

In view of the in vitro data, the limited antitumor effect of bisantrene in phase II clinical trials has been disappointing. One hundred and twenty-one patients with metastatic renal cell carcinoma have been treated with bisantrene according to various dosage schedules.[4] Only two partial responses have been observed. Both of these patients had been given bisantrene (270 mg/m^2) every 3 weeks. Alberts et al (3), using a weekly × 3 schedule similar to ours, observed one partial response in 16 patients. Our observation of only one partial response and two mixed responses in 20 evaluable patients is further indication of the very limited activity of bisantrene in this tumor type.

Based on our results and those reported in the literature, we do not recommend further investigation of bisantrene in renal cell carcinoma.

REFERENCES

1. Investigators Brochure. CL 216, 942 9, 10-anthracenedicarboxyaldehyde bis [(4,5-dihydro-1H-imidazol-2-yl)hydrazone]dihydrochloride. Pearl River, NY, American Cyanamid Company, Medical Research Division, Oct 1979.
2. SAROSDY MF, LAMM DL, RADWIN HM, ET AL. Clonogenic assay and *in vitro* chemosensitivity testing of human urologic malignancies. Cancer 50:1332–1338, 1982.
3. ALBERTS DS, MACKEL C, POCELINKO R, ET AL. Phase I clinical investigation of 9, 10-anthracenedicarboxaldehyde bis[(4,5-dihydro-1H-imidazol-2-yl)hydrazone]dihydrochloride with correlative in vitro human tumor clonogenic assay. Cancer Res 42:1170–1175, 1982.

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[4] Personal communication. American Cyanamid Co, Medical Research Division, Sept 1982.

Effects of Cytotoxic Agents on Iron Metabolism

Sethi et al (1) recently reported increases of serum iron concentrations in monkeys and humans administered vincristine, vinblastine, or vindesine. They speculate as to the mechanism of this effect, and state that, to their knowledge, "this is the first report in the literature on the effect of cytotoxic drugs on iron metabolism."

We wish to point out that effects of cytotoxic agents on iron metabolism have been known for some time and have been extensively studied. In 1958, Bertinchamps et al (2) demonstrated increased plasma iron concentrations and delayed plasma radioiron clearance after the administration of mechlorethamine. Detailed studies of the effect of HN_2 on the distribution of radioiron in human subjects were reported by Lane et al (3). These investigations demonstrated that after administration of HN_2, there was a marked diminution of uptake of radioiron by the bone marrow, elevation of the serum iron concentration, diminished rbc uptake of radioiron, and increased uptake of radioiron by the liver and spleen. These ferrokinetic changes were associated with reticulocytopenia. The phenomenon was also extensively studied in dogs (4). Subsequently, Alfrey et al (5) reported similar effects resulting from the administration of methotrexate, 5-FU, dactinomycin, hydroxyurea, and galactoflavin. In each instance, hyperferremia and a similar pattern of ferrokinetic changes were observed in association with suppressed erythropoiesis. Although Sethi et al state that the rbc count in the rhesus monkeys did not change and that the hematocrit and hemoglobin values were also not affected, they did not report on the reticulocyte counts. The drugs they studied are all known to produce reticulocytopenia, and we consider this inhibition of erythropoiesis to be the likely explanation for the observed elevations in serum iron concentrations.

REFERENCES

1. SETHI VS, SHIHABI Z, and SPURR CL. Increase of serum iron concentrations in rhesus monkeys and humans on administration of vincristine, vinblastine, or vindesine. Cancer Treat Rep 68:933-936, 1984.
2. BERTINCHAMPS A, KENIS Y, and TAGNON HJ. Effect of administration of HN_2 on utilization of plasma iron. Cancer 11:117-121, 1958.
3. LANE M, BERLIN NI, and MENGEL CE. The effect of nitrogen mustard on the distribution of radioiron in man. Cancer 37:322-328, 1964.
4. LANE M, KARJALA RJ, LIEBELT RA, ET AL. The effects of nitrogen mustard on iron distribution and localization in the dog. Cancer 18:1265-1277, 1965.
5. ALFREY CP, JR, LANE M, and KARJALA RJ. Modification of ferrokinetics in man by cancer chemotherapeutic agents. Cancer 19:428-432, 1966.

Montague Lane[*]
Clarence P. Alfrey, Jr
Frank E. Smith[1]

August 9, 1984

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[1] Division of Clinical Oncology, Department of Pharmacology, Baylor College of Medicine, Houston, TX.

[*] *Reprint requests to:* Montague Lane, MD, Division of Clinical Oncology, Department of Pharmacology, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030.

Reply

Catharanthus antitumor alkaloids were discovered in the early 1960s, and our literature search obviously missed the important contributions of Bertinchamps et al (1), Lane et al (2,3), and Alfrey et al (4) on the effect of mechlorethamine and other chemotherapeutic agents (methotrexate, 5-FU, dactinomycin, hydroxyurea, and galactoflavin) on iron metabolism. In view of these and our preliminary findings, it would seem important to study the effect of other anticancer drugs with diverse structures and mechanisms of action on iron metabolism.

REFERENCES

1. BERTINCHAMPS A, KENIS Y, and TAGNON HJ. Effect of administration of HN_2 on utilization of plasma iron. Cancer 11:117-121, 1958.
2. LANE M, BERLIN NI, and MENGEL CE. The effect of nitrogen mustard on the distribution of radioiron in man. Cancer 37:322-328, 1964.

3. LANE M, KARJALA RJ, LIEBELT RA, ET AL. The effects of nitrogen mustard on iron distribution and localization in the dog. Cancer 18:1265-1277, 1965.
4. ALFREY CP, JR, LANE M, and KARJALA RJ. Modification of ferrokinetics in man by cancer chemotherapeutic agents. Cancer 19:428-432, 1966.

V. Sagar Sethi[1, *]
Z. Shihabi[2]
Charles L. Spurr[2]

October 18, 1984

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[1] Department of Chemistry, University of Texas at El Paso.

[2] Department of Pathology and Medicine, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC.

[*] *Reprint requests to:* Dr. V. Sagar Sethi, Department of Chemistry, University of Texas, El Paso, TX 79968.

Acute Cardiac Failure From Doxorubicin Cardiotoxicity Provoked in Relation to Second-Look Laparotomy

It is well established that anthracycline antitumor antibiotics induce a dose-dependent, progressive, irreversible cardiomyopathy which ultimately may terminate in cardiac failure (1,2). The incidence of cardiac failure increases abruptly if the total cumulative dose is > 550 mg/m^2 of body surface area (1,2). More than 30% reduction in QRS voltage in the limb leads of the ECG significantly correlates with cardiomyopathy in patients with anthracycline-induced cardiac failure (3). Other indicators are cardiomegaly on chest x-ray (1,2) and decrease in radionuclide ejection fraction (4). The final diagnosis of anthracycline cardiomyopathy is established by endomyocardial biopsy (5). Acute cardiac failure due to anthracycline cardiotoxicity and elicited by general anesthesia and major surgery has not been reported previously.

During a 4-year period, 60 second-look laparotomies were performed at the Finsen Institute on patients with ovarian cancer after treatment with $\geqslant 500$ mg/m^2 of doxorubicin (highest cumulative dose, 580 mg/m^2). Doxorubicin was given intermittently in a dose of 30 mg/m^2 iv on Days 1 and 8 every 4 weeks as part of a combination chemotherapy regimen that also included cyclophosphamide and 5-FU. None of the patients had a prior history of heart disease, and the median age was 56 years (range, 20-70). ECG and chest x-ray were performed routinely before chemotherapy was started, regularly during the treatment, and prior to surgery, as well as part of the follow-up controls after discontinuation of chemotherapy.

Two patients (3.3%; 95% confidence limits, 0.4%-11.5%) developed acute cardiac failure in relation to the second-look laparotomy. None of the patients had clinical symptoms or signs of cardiac insufficiency prior to surgery.

CASE REPORTS

Patient 1

A 58-year-old woman with serous cystadenocarcinoma of the ovary was treated for 9 months with combination chemotherapy (cyclophosphamide, doxorubicin, and 5-FU). After a total dose of 530 mg/m^2 of body surface area, a second-look laparotomy was performed. During this operation, the patient developed a drop in blood pressure, cyanosis, and signs of pulmonary stasis. Pulmonary embolism was suspected, but a lung scan was normal. Two weeks later, the patient again developed signs of acute cardiac failure with dyspnea, cyanosis, tachycardia, and unmeasurable blood pressure in relation to fever (urinary infection). ECG showed reduction (60%) in QRS volt amplitude (sum of six limb leads) and sinus tachycardia. The heart diameter measured on chest x-ray was increased from 12.5 cm before initiation of chemotherapy to 15.5 cm at the time of second-look operation (thoracic diameter, 28 cm). At this time, the condition was interpreted as doxorubicin cardiomyopathy and was effectively treated with oxygen, sympathomimetic amines, loop diuretics, and digoxin. Radionuclide angiocardiography showed a left ventricular ejection fraction of 18% at rest (normal $\geqslant 50\%$) and no increase during exercise. The diagnosis of severe doxorubicin cardiomyopathy was supported by endomyocardial biopsy, showing widespread myocyte degeneration and interstitial fibrosis.

Patient 2

A 57-year-old female with ovarian cancer was treated for 12 months with combination chemotherapy (cyclophosphamide, doxorubicin, and 5-FU). The total doxorubicin dose was 500 mg/m^2. In relation to second-look laparotomy, the patient developed acute pulmonary edema and hypotension, which was treated adequately with oxygen, loop diuretics, and digoxin. ECG showed sinus tachycardia but no reduction in QRS voltage. Chest x-ray showed a minor increase in heart size. Radionuclide ejection fraction at rest was 33%, with only minor increase during exercise (38%). The diagnosis of moderate doxorubicin cardiomyopathy was supported by endomyocardial biopsy.

Judged from a left-sided heart catheterization with ventriculography and aortography, there was in both cases no suspicion of concomitant coronary artery disease affecting the major epicardial vessels. Also, ECG showed no evidence of myocardial ischemia or infarction.

In addition, two patients (3.3%) developed symptoms and signs of moderate cardiac insufficiency 2 and 8 months after the second-look laparotomy (3 and 10 months after cessation of doxorubicin, respectively). Both patients revealed increases in heart size upon chest x-ray. From a retrospective judgement, the latter patient had a 28% reduction in QRS voltage and multiple ventricular extrasystoles that were time-related to the second-look laparotomy. Among the remaining 56 patients who did not develop cardiac insufficiency, none developed significant ECG changes or increases in heart size.

It is concluded that laparotomy may be a special hazard for patients treated with high doses of doxorubicin ($\geqslant 500$ mg/m^2). As this drug is frequently incorporated in combination chemotherapy for the treatment of ovarian cancer, and as these patients are potentially free of malignant disease, the clinician should evaluate the patients carefully before they are subjected to major surgery. Special attention should be paid to QRS voltage reduction, increase in heart size on chest x-ray, and a subnormal radionuclide ejection fraction. In cases of doubt, heart catheterization with endomyocardial biopsy should be performed prior to surgery.

When anesthesia and major surgery are mandatory in patients with reduced cardiac reserve due to anthracycline cardiomyopathy, prophylactic digitalization is advocated. The patient should be carefully monitored during surgery and the first postoperative days.

Oncologists, anesthesiologists, and surgeons should be aware of potential complications from chronic anthracycline cardiotoxicity without prominent clinical symptoms.

REFERENCES

1. LEFRAK EA, PITHA J, ROSENHEIM S, ET AL. A clinicopathologic analysis of adriamycin cardiotoxicity. Cancer 32:302-314, 1973.
2. VON HOFF DD, LAYARD MW, BASA P, ET AL. Risk factors for doxorubicin-induced congestive heart failure. Ann Intern Med 91:710-717, 1979.
3. MINOW RA, BENJAMIN RS, LEE ET, ET AL. QRS voltage change with adriamycin administration. Cancer Treat Rep 62:931-934, 1978.
4. ALEXANDER J, DAINIAK N, BERGER HJ, ET AL. Serial assessment of doxorubicin cardiotoxicity with quantitative radionuclide angiocardiography. N Engl J Med 300:278-293, 1979.
5. BRISTOW MR, MASON JW, BILLINGHAM ME, ET AL. Doxorubicin cardiomyopathy: evaluation by phonocardiography, endomyocardial biopsy, and cardiac catheterization. Ann Intern Med 88:168-175, 1978.

Kristian Aabo[1,*]
Svend A. Mortensen[2]
Torsten Jonsson[1]
Heine H. Hansen[1]

November 15, 1984

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[1] Departments of Chemotherapy (*K. Aabo* and *H. H. Hansen*) and Anesthesiology (*T. Jonsson*), The Finsen Institute, Copenhagen, Denmark.

[2] Department of Cardiology, Rigshospitalet, Copenhagen, Denmark.

[*] *Reprint requests to:* Kristian Aabo, MD, Department of Chemotherapy, The Finsen Institute, 49 Strandboulevarden, DK-2100 Copenhagen, Denmark.

Successful Continuation of Cisplatin Therapy After Partial Recovery From Acute Renal Failure

Although renal damage resulting in decreased creatinine clearance and magnesium wasting is a well-characterized potential complication of conventional-dose platinum therapy, acute renal failure requiring hemodialysis after conventional doses of cisplatin (100-120 mg/m^2) is uncommon (1). Administration of cisplatin at more than the normal doses (200 mg/m^2) may result in transient and sporadic elevations of serum creatinine, but has not been reported to cause acute renal failure which requires hemodialysis, and the serum creatinine usually returns to normal within 2-3 weeks. During the administration of conventional-dose cisplatin (100 mg/m^2) to a patient with seminoma, acute renal failure requiring hemodialysis developed after the first course of chemotherapy. After partial return of renal function, the patient was given three additional cycles of cisplatin/vinblastine/doxorubicin (including cisplatin at 100 mg/m^2) every 3 weeks without a recurrence of further deterioration in renal function.

CASE REPORT

A 38-year-old man was diagnosed as having seminoma, clinical stage A, and was treated with retroperitoneal radiation after transinguinal orchiectomy in 1982. In December 1983, he presented with a 4-month history of left chest pain. Chest x-ray and chest and abdominal computerized tomographic scans revealed a large posterior mediastinal mass and retroperitoneal lymphadenopathy without evidence of obstructive uropathy. A needle biopsy of the mediastinal mass confirmed recurrence of seminoma. Alpha fetoprotein and beta-human chorionic gonadotropin were negative.

On February 2, 1984 (designated as Day 1), the patient was started on chemotherapy with cisplatin (100 mg/m^2), vinblastine (0.3 mg/kg), and bleomycin (30 units). Pretreatment serum creatinine was 0.8 mg/dl and the creatinine clearance was 120 ml/minute. Cisplatin (100 mg/m^2) was given in 5% dextrose in one-half normal saline as an infusion over 24 hours, with iv (125 ml/hour) and oral (about 120 ml/24 hours) hydration which sustained a urine output > 150 ml/hour for > 48 hours. A diuretic was not used, because urine output was adequate. He was given allopurinol (to prevent hyperuricemia) and hydromorphone (for pain), and lithium carbonate (300 mg twice a day) was administered as part of an experimental treatment protocol designed to evaluate its impact on attenuating the myelosuppression which usually attends the cisplatin/vinblastine/bleomycin combination chemotherapy. Serum creatinine on Day 4 was 1.1. The patient was discharged on Day 5 in good physiological condition. On Day 9, he was given a second dose of bleomycin (30 units iv). The result of a serum creatinine analysis, which became available after the bleomycin injection, showed that the serum creatinine and BUN had risen to 5.0 and 38 mg/dl, respectively. The patient was feeling well and was not oliguric at the time. On Day 11, the patient presented with a fever and *Escherichia coli* bacteremia without hypotension. At that time, he had serum creatinine of 9.2 mg/dl, BUN of 57 mg/dl, uric acid of 4.7 mg/dl, and urine output < 20 ml/hour. Renal ultrasound was normal. Disseminated intravascular coagulation screen was negative. Allopurinol and lithium were discontinued and antibiotic therapy, including tobramycin, was started. Serum creatinine reached 9.7 mg/dl, so hemodialysis was initiated on Day 13 and repeated on Days 14 and 16. Renal biopsy was considered but was not done. The patient's renal function subsequently improved gradually, with serum creatinine levels of 6.3 mg/dl on Day 21, 3.5 mg/dl on Day 25, and 1.9 mg/dl with a creatinine clearance of 45 ml/minute on Day 20. During this time, the patient manifested signs of severe pulmonary and cutaneous bleomycin toxicity.

From March 8, 1984 (Day 36) to April 24, 1984, the patient was given three cycles of chemotherapy with cisplatin, vinblastine, and doxorubicin every 3 weeks. Each time, cisplatin (100 mg/m^2) was given in 250 ml of 3% saline over 1 hour after furosemide (40 mg iv), with 5% dextrose in normal saline plus 20 mEq of KCl/L at 250 ml/hour as prehydration for 12 hours and posthydration for 24 hours. This chemotherapy was complicated by neutropenia and fever, but the patient's renal function has remained stable, with serum creatinine of 1.2-1.6 mg/dl and creatinine clearance of 45-50 ml/minute on repeated measurements. The patient's tumor has markedly decreased in size, and he is currently doing well.

DISCUSSION

The acute renal failure which complicated the first cycle of cisplatin, vinblastine, and bleomycin therapy in this patient was undoubtedly due in part to cisplatin. Other medications that the patient received (allopurinol, lithium carbonate, and hydromorphone) may have renal side effects, but are not known to cause acute renal failure requiring hemodialysis, and no unusual toxicity has occurred in other patients on the same lithium carbonate treatment protocol. Although his *E. coli* sepsis occurred during the evolution of the acute renal failure, the patient was documented to have normal blood pressure and urine output, and was afebrile at the time the serum creatinine had risen to 5 mg/dl. Thus, the onset of acute renal failure had preceded the episode of sepsis and the use of aminoglycoside. Furthermore, acute renal failure was not due to obstructive uropathy, and the patient's kidneys were outside the radiation field during his course of retroperitoneal radiation in 1982. During his first course of chemotherapy, the patient remained well-hydrated with good urine output, and did not receive other nephrotoxic agents such as iv dye or aminoglycosides.

We believe this is the first instance in which cisplatin therapy has been administered in full doses after partial recovery from acute renal failure requiring hemodialysis. Stoter et al (2) reported two cases and Einhorn and Donohue (3) reported one case of acute renal failure requiring hemodialysis among a total of 90 patients who received chemotherapy with cisplatin, bleomycin, and vinblastine. In these three patients, subsequent courses of cisplatin were not given or were given in reduced doses with delayed intervals. In contrast, our patient was subsequently given three more cycles of chemotherapy with the same dose of cisplatin at 100 mg/m^2 every 3 weeks, without further decrease in creatinine clearance.

The three courses of cisplatin which followed partial recovery from renal failure were delivered in the presence of 3% saline and very vigorous hydration to reduce the probability of a recurrence of cisplatin-induced renal failure. In a series of patients treated with high-dose cisplatin (200 mg/m^2), no renal toxicity other than hypomagnesemia was observed, using the 3% hypertonic saline bolus administration of cisplatin at 40 mg/m^2 iv daily on Days 1-5 every 3 weeks (4). In another report by Ozols et al (5), sporadic mild elevations in serum creatinine were observed following the administration of high-dose cisplatin (200 mg/m^2), but these episodes were reversible and did not recur with subsequent administration. The experience of Ozols et al as well as the experience described in this report strongly suggest that the occurrence of cisplatin-induced renal failure is very often reversible and need not render a patient ineligible for subsequent cisplatin therapy, providing adequate recovery of renal failure has occurred.

REFERENCES

1. BLACHLEY JD, and HILL JB. Renal and electrolyte disturbances asso-

ciated with cisplatin. Ann Intern Med 95:628-632, 1981.

2. STOTER G, SLEIJFER D, VENDRIK CPJ, ET AL. Combination chemotherapy with *cis*-diammine-dichloro-platinum, vinblastine and bleomycin in advanced testicular non-seminoma. Lancet 1:941-945, 1979.
3. EINHORN LH, and DONOHUE J. *Cis*-diamminedichloroplatinum, vinblastine and bleomycin combination chemotherapy in disseminated testicular cancer. Ann Intern Med 87:293-298, 1977.
4. OZOLS RF, DEISSEROTH AB, JAVADPOUR N, ET AL. Treatment of poor prognosis nonseminomatous testicular cancer with a "high dose" platinum combination chemotherapy regimen. Cancer 51:1803-1807, 1983.
5. OZOLS RF, CORDEN BJ, JACOB J, ET AL. High-dose cisplatin in hypertonic saline. Ann Intern Med 100:19-24, 1984.

Dan C. Wong
Albert B. Deisseroth[1,*]

November 23, 1984

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[1] University of California at San Francisco Hematology/Oncology Unit, Veterans Administration Medical Center.

* *Reprint requests to:* Dr. Albert B. Deisseroth, University of California at San Francisco Hematology/Oncology Unit (111H), Veterans Administration Medical Center, 4150 Clement St, San Francisco, CA 94121.

Combination of Lomustine and Cimetidine in the Treatment of a Patient With Malignant Glioblastoma: A Case Report

The nitrosoureas have produced significant bone marrow suppression and are often prescribed with other drugs that cause hematologic abnormalities, an example of which is cimetidine. Selker et al (1) described the leukopenic effect of simultaneous administration of one of these nitrosoureas, carmustine, with cimetidine in a small group of patients. The following is a case where a different nitrosourea, lomustine, in combination with cimetidine, resulted in clinically significant leukopenia and neutropenia, only when both agents were used concomitantly.

CASE REPORT

A 55-year-old white male with highly malignant glioblastoma was admitted to the hospital on an emergency basis 53 days (120 mg) and 16 days (160 mg) after lomustine was given. Bronchopneumonia and an oral candida infection resulting from leukopenia (900 cells/mm^3) and neutropenia (< 5%) were treated with the appropriate antibiotics. A bone marrow aspirate revealed a shift to the left with little maturation of the cells of the myelocytic stage. This was consistent with a drug-induced neutropenia.

The patient's past medications included cimetidine (300 mg every 6 hours), dexamethasone (12 mg every 6 hours), phenytoin (100 mg three times a day), and phenobarbital (30 mg three times a day). No hematologic abnormalities had been detected for 2 months prior to the lomustine dose. On Day 55 after the 120-mg lomustine dose, the cimetidine was discontinued. The leukopenia and neutropenia rapidly reversed, reaching normal ranges (5300/mm^3 leukocytes with 51% neutrophils) by Day 68 after the 120-mg lomustine dose.

The nadir from the 160-mg lomustine dose occurred between 46 and 49 days after this dose, but was much less severe (4000/mm^3 leukocytes with 72% neutrophils). The patient was discharged shortly thereafter.

The leukocyte count nadirs occurring in our patient matched the values previously obtained in the general population, occurring between Days 42 and 56. There was an absence of any hematologic abnormalities prior to lomustine (120 mg) while the patient was receiving cimetidine, and there was minimal leukocyte depression after lomustine (160 mg) while he was not receiving cimetidine. This clearly indicates that the combination of lomustine and cimetidine potentiated the leukopenic and neutropenic effects of lomustine in our patient.

A previous report (1) indicated that this type of drug interaction was suspected when carmustine was used in conjunction with cimetidine. Indeed, cimetidine alone has produced leukopenia, neutropenia, thrombocytopenia, pancytopenia, and agranulocytosis, with an overall incidence of 2.3 patients per 100,000 (2). In view of these data, it appears that the combination of lomustine and cimetidine should be used cautiously.

REFERENCES

1. SELKER RG, MOORE P, and LODOLCE D. Bone marrow depression with cimetidine plus carmustine. N Engl J Med 299:834, 1978.
2. DAVIS TG, PICKETT DL, and SCHLOSSER JH. Evaluation of a worldwide spontaneous reporting system with cimetidine. JAMA 243:1912-1914, 1980.

William A. Hess[1,*]
Paul L. Kornblith[2]

November 30, 1984

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[1] Pharmacy Department, Clinical Center, National Institutes of Health, Bethesda, MD.

[2] Surgical Neurology Branch, Intramural Research Program, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, MD.

* *Reprint requests to:* William A. Hess, Pharmacy Department, Clinical Center, National Institutes of Health, Bldg 10, Rm 1S-257, Bethesda, MD 20205.

Acute ECG Changes During Cyclophosphamide Infusion in a Patient With Bronchogenic Carcinoma

ECG changes following administration of some cytotoxic agents are a well-documented phenomenon, but, to our knowledge, have not been described for therapeutic ranges of cyclophosphamide or other alkylating agents. This report documents a Prinzmetal's angina-like phenomenon in a patient during the infusion of cyclophosphamide as treatment for bronchogenic carcinoma.

CASE REPORT

A 58-year-old man without previous history of heart disease was admitted to the Chest Department because of weight loss and a cough of 4 months' duration. A chest x-ray showed a mass of 8 cm in diameter in the apical segment of the right upper lobe. A fine-needle aspiration of the mass revealed poorly differentiated malignant cells. The patient received radiotherapy (4000 rad) in two courses to the upper field of the right lung and an infusion of cyclophosphamide (30 mg/kg) once in 3 weeks. A control chest x-ray after a few months showed a significant reduction of the mass. Because of the good response, cyclophosphamide treatment was continued for 2 years. This treatment was well tolerated by the patient, who had only mild nausea and vomiting during the infusion, which responded to iv injection of 10 mg of metoclopramide. At his last treatment, nausea and vomiting occurred 2 minutes after the infusion was started with 1900 mg of cyclophosphamide in 150 ml of NaCl 0.9%. This time, the vomiting continued even after metoclopramide had been slowly injected. The patient was covered with cold sweat, and about 4 minutes after the infusion had been started, the radial pulse was not palpable. There was no dyspnea or chest pain. At that time, the patient had already received about 300–400 mg of cyclophosphamide. The cyclophosphamide was immediately stopped, and limb leads 1, 2, and 3 of the ECG were registered. Severe ST elevation appeared in L_2, L_3 in the sinus and the ventricular premature beats (fig 1A). Runs of ventricular premature beats were followed by bradycardia (fig 1B), and 0.5 mg of atropine sulfate was therefore injected iv. About 7 minutes after starting the infusion, sinus rhythm of 105 beats/minute appeared with third-degree atrioventricular (AV) block (fig 1C), followed by second-degree AV block (fig 1D), while the ST normalized gradually. At this time, the radial pulse was palpable again, and a complete ECG performed after another 2 minutes was normal (limb lead 3 is demonstrated in fig 1E). The patient was monitored for 48 hours. During this period, no abnormalities in ECG occurred, and there was no serum enzyme elevation. The remainder of the cyclophosphamide solution was examined by the pharmaceutic manufacturer and no irregularities were found. Cyclophosphamide treatment was replaced by methotrexate. Nine months later, the patient died due to dissemination of the malignant disease. Until his death, there was no further evidence of heart disease. Autopsy was refused.

DISCUSSION

Acute ECG changes have been reported during the infusion of 5-FU and after the injection of vincristine. It is also well known that cyclophosphamide may induce severe cardiomyopathy when given in supralethal doses to candidates for bone marrow transplantation (1). A case of reversible pulmonary edema following cyclophosphamide treatment has also been described (2). However, it was attributed to allergic reaction and no ECG was performed. As far as we know, our patient represents the first reported case of severe ECG changes during administration of a conventional therapeutic dosage of this drug. The transient ST elevation and the severe rhythm disturbance in the ECG, as well as the absence of blood enzyme elevation, have similarities to the so-called "Prinzmetal's angina" or "variant angina" (3). This dis-

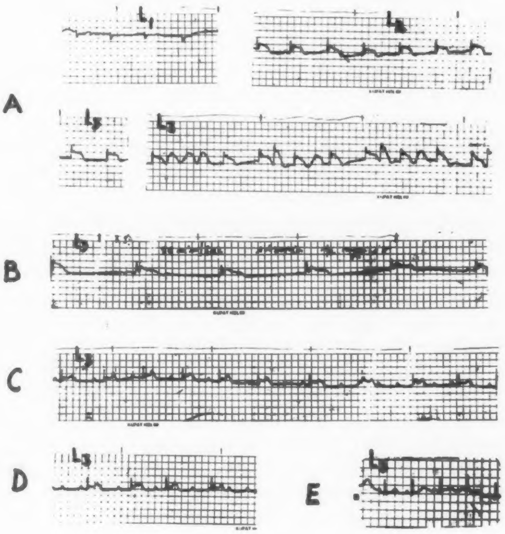

FIGURE 1.—**A:** ECG changes following collapse of the patient. In limb leads 2 and 3 (L_2, L_3), right-axis deviation, ST elevation, and runs of ventricular tachycardia are seen (paper speed 25 mm/sec). **B:** Sinus bradycardia after runs of ventricular tachycardia (paper speed 50 mm/sec). **C:** Third-degree AV block after 0.5 mg of atropine sulfate iv (paper speed 25 mm/sec). **D:** Wenckebach's phenomenon (paper speed 25 mm/sec). **E:** Normal ECG (paper speed 25 mm/sec).

ease is attributed to coronary spasm and is not dependent on increased myocardial oxygen consumption. Indeed, chest pain is an essential part of this syndrome, but typical ECG changes caused by the same mechanism have also been described without chest pain (4). Paradoxically, this sort of angina may be induced by sympathomimetic drugs, as well as by parasympathomimetic drugs, and is related to increased vagal tonus (5). Therefore, in the pathogenesis, an interrelation between the sympathicus and parasympathicus system was considered. We assume that the ECG changes in our patient are due to increased vagal tonus (vomiting, bradycardia) induced by cyclophosphamide. It seems to us that more attention should be paid to coronary side effects in cases of acute reaction to this drug.

REFERENCES

1. MILLIS BA, and ROBERTS EQ. Cyclophosphamide induced cardiomyopathy: a report of two cases and a review of the English literature. Cancer 43:2223-2226, 1979.
2. MAXWELL MB. Reversible pulmonary edema following cyclophosphamide treatment. JAMA 229:137-138, 1974.
3. PRINZMETAL M, KENNAMER R, MERLISS R, ET AL. Angina pectoris 1. A variant form of angina pectoris. Am J Med 27:375-388, 1959.
4. ROBERTSON D, ROBERTSON RM, MIES AS, ET AL. Variant angina pectoris: investigation of indexes of sympathetic nervous system function. Am J Cardiol 43:1080-1085, 1979.
5. YASUE H, TOUYAMA M, SHIMAMOTO M, ET AL. Role of autonomic nervous system in the pathogenesis of Prinzmetal's variant form of angina. Circulation 50:534-539, 1974.

Joshua Shachor
Bruno Beker
Yaacov Geffen
Israel Bruderman[1,*]

December 3, 1984

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[1] Chest and Cardiology Departments, Meir General Hospital, Kfar-Saba, Israel.

[*] *Reprint requests to:* Israel Bruderman, MD, Chest Department, Meir General Hospital, Kfar-Saba 44281, Israel.

Local Toxicity From Iv Administration of Baker's Antifol

In a recent review, local complications of iv chemotherapy administration were classified as hypersensitivity reactions, chemical irritation, and extravasation-induced tissue injury (1). A list of drugs known to cause these reactions was provided. We have consistently observed local toxicity associated with the administration of iv Baker's antifol (triazinate, NSC-139105), and feel that this drug should be included among the drugs causing such side effects.

Baker's antifol was given weekly as a single agent in 1-hour infusions (300-600 mg/m^2) diluted to 100 ml in normal saline. Most of the 16 patients treated to date have developed hyperpigmentation over the veins in the infused arm, developing within 1-2 weeks. The reaction also involved most of the tributaries fed by the vein; in addition, retrograde involvement often occurred to the base of the hand. Initially, the veins appeared reddened, but no tenderness or discomfort was reported. Redness was often noted immediately after onset of the infusion. Hyperpigmentation ensued and persisted; the veins remained patent and could be used for further chemotherapy administration. Necrosis did not occur. Our clinical impression was a hypersensitivity reaction with post-inflammatory hyperpigmentation. Despite lack of symptoms, many patients were distressed at the appearance of the veins.

After obtaining informed consent from two patients, punch biopsies of hyperpigmented areas were obtained, processed by standard methods for light microscopy and stained with hematoxylin and eosin. Examination revealed melanin pigment incontinence with pigment-laden macrophages in the dermis, and mild dermal fibrosis with a chronic perivascular inflammatory infiltrate consisting of lymphocytes in both biopsies, and, additionally, eosinophils in one. These findings confirm the clinical impression of a hypersensitivity-type reaction.

In addition, four of our patients developed transient urticarial reactions limited to the skin over the infused veins. There was minimal pruritis, and pretreatment with Benadryl had little effect on its formation. One of these patients developed an asthmatic reaction during the sixth infusion which responded to hydrocortisone and Benadryl.

One of our patients has had extravasation of about 10-20 mg of Baker's antifol into the dorsum of the hand. No specific treatment was given and there were no adverse sequelae, suggesting that extravasation necrosis may not occur with this drug.

Baker's antifol is a dihydrofolate reductase inhibitor whose biochemical pharmacology and spectrum of clinical toxicity differ from that of methotrexate (2). Systemic hypersensitivity with cutaneous reactions characteristic of erythema multiforme are common and sometimes severe (3,4), but local hypersensitivity reactions have not been previously reported. These toxic effects may be mediated by histamine, since histaminase inhibition is a known property of Baker's antifol and other newer antifol antimetabolites (5). Since we have regularly observed such toxicity, we feel that it probably occurs more frequently than is recognized. It is important because of its adverse cosmetic effect and resulting patient distress. Mention of this side effect should be included in information given to patients starting treatment with Baker's antifol.

REFERENCES

1. HARWOOD KV, and AISNER J. Treatment of chemotherapy extravasation: current status. Cancer Treat Rep 68:939-945, 1984.
2. TOWNSEND JG, JAIN RK, and CASHMORE AR. In vivo pharmacokinetics of triazinate in L-1210 and W-256 cells. J Pharm Sci 71:1102-1105, 1982.
3. RODRIGUEZ V, GOTTLIEB J, BURGESS MA, ET AL. Phase I studies with Baker's antifol (BAF) (NSC-139105). Cancer 38:690-694, 1976.
4. RODRIGUEZ V, RICHMAN SP, BENJAMIN RS, ET AL. Phase 2 studies with Baker's antifol in solid tumors. Cancer Res 37:980-983, 1977.
5. DUCH DS, EDELSTEIN MP, and NICHOL CA. Inhibition of histamine-metabolizing enzymes and elevation of histamine levels in tissues by lipid-soluble anticancer folate antagonists. Mol Pharmacol 18:100-104, 1980.

Steven H. Krasnow[1,2,*]
Robert H. Patterson[1,2]
Betty Fischmann[1,3]
Mario A. Eisenberger[4]
Anita Johnston-Early[1]
Marc L. Citron[1,3,5]
Martin H. Cohen[1,2]

December 10, 1984

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[1] Departments of Medicine (*S. H. Krasnow, B. Fischmann, M. L. Citron,* and *M. H. Cohen*), Pathology (*R. H. Patterson*), and Nursing (*A. Johnston-Early*), Veterans Administration Medical Center, Washington, DC.

[2] Departments of Medicine (*S. H. Krasnow* and *M. H. Cohen*) and Pathology (*R. H. Patterson*), George Washington University Medical Center, Washington, DC.

[3] Department of Medicine, Georgetown University Medical Center, Washington, DC.

[4] Clinical Investigations Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

[5] *Present address:* Oncology Section, Long Island Jewish-Hillside Medical Center, New Hyde Park, NY.

[*] *Reprint requests to:* Steven H. Krasnow, MD, Veterans Administration Medical Center, Ward 2C North, 50 Irving St, NW, Washington, DC 20422.

Fifth Annual Symposium of Fine Needle Aspirations

Santa Barbara, California, August 17–23, 1985

This symposium, sponsored by the University of California and scheduled to be held at the Biltmore Resort, will provide a comprehensive introduction to both biopsy techniques and interpretive criteria. Diagnostic complexities will also be examined through a balanced program of didactic and microscopic sessions. Due to the intensive nature of the program, attendance will be limited to 50 pathologists. Microscopes are requisite. The program meets the criteria for 20 hours of AMA/CMA/ACCME Category 1 credit for physicians.

For further information, contact: Extended Programs in Medical Education, Room 569-U, University of California, San Francisco, CA 94143 (telephone: 415-666-4251).

National Conference on Pediatric Brain Tumors

Minneapolis, Minnesota, August 21–23, 1985

The Pediatric Brain Tumor Task Force at the University of Minnesota has developed this conference, which will focus on neurology, radiology, and neurosurgery. The course is accredited for 16 hours in Category 1 by the American Medical Association. The fee is $250.

For further information, contact: Continuing Medical Education, University of Minnesota, Box 293, 420 Delaware St SE, Minneapolis, MN 55455 (telephone: 612-373-8012).

Fourteenth International Cancer Congress

Budapest, Hungary, August 21–27, 1986

For further information about this International Union Against Cancer (UICC) meeting, contact: Congress Bureau MOTESZ, P. O. Box 32, H-1361 Budapest, Hungary [in North America: Crimson Travel Service, 39 John F. Kennedy St, Cambridge, MA 02138 (telephone: 1-800-343-2042)].

Fourth World Conference on Lung Cancer

Toronto, Canada, August 25–30, 1985

An anticipated 2500 members of the international medico-scientific community with special interest in lung cancer will gather to share new ideas, research results, and experiences. Under the auspices of the International Association for the Study of Lung Cancer, the conference will be a combination of panels, symposia, lectures, workshops, ciné clinics, and exhibits.

Topics addressed will be prevention, epidemiology, respiratory carcinogenesis, early diagnosis and staging pathology, surgery, radiotherapy, chemotherapy, immunotherapy, cellular kinetics, tumor markers, and imaging. The site of the sessions and exhibit will be the Metro Toronto Convention Center.

For further information, contact: Dr. Ronald Feld, Secretary General, The IV World Conference on Lung Cancer, c/o Secretariat Office, 342 MacLaren St, Ottawa, Ontario, Canada K2P OM6.

XVI Congress of the International Academy of Pathology and Seventh World Congress of Academic and Environmental Pathology

Vienna, Austria, August 31–September 5, 1986

For further information, contact: Congress Office, Institute for Pathologische Anatomie, 4 Spitalgasse, A-1090 Vienna, Austria [in North America: Crimson Travel Service, 39 John F. Kennedy St, Cambridge, MA 02138 (telephone: 1-800-343-2042)].

New Avenues in Developmental Cancer Chemotherapy

London, England, September 4–5, 1985

This eighth annual Bristol-Myers Symposium on Cancer Research will be organized by Dr. Ken R. Harrap, Department of Biochemistry, Institute of Cancer Research, Surrey, England.

More than 25 speakers from the United States, United Kingdom, Europe, and Israel will discuss a variety of subjects relating to new techniques for designing anticancer drugs. Topics include: how an understanding of oncogenes may provide a basis for selective drug design; how monoclonal antibodies can be used to target drugs; the role of mathematical modeling in drug design and utilization; the biochemical modulation of drug activity; initiation of cell differentiation; and new drugs in development.

The symposium will be held at the Kensington Town Hall. A registration fee of £55 should be made payable to Institute of Cancer Research: Bristol-Myers Symposium. The deadline for receipt of registration forms is *July 1, 1985*.

For further information, contact: Ms. Ann Robinson, Bristol-Myers Symposium, Institute of Cancer Research, Block E, Clifton Ave, Belmont, Sutton, Surrey SM2 5PX, England (telephone: 01-643-8901).

Legal Issues in Medical Practice

San Francisco, California, October 4–5, 1985

The Department of Family Medicine of the University of California and the American College of Legal Medicine are sponsoring this course designed to familiarize clinicians with recent legislative measures which will affect medical practice and to discuss areas of ongoing legal and ethical concern. Topics include: durable power of attorney for health care, legal obligations relating to the care of the elderly, "no-code" orders and the withdrawal of life support; hospital staff privileges; legal bases for license revocation; patient communication; and the litigious patient.

The course should be of particular interest to physicians in high-risk specialties but may also be of value to hospital administrators, risk managers, and quality assurance coordinators. The program will meet the criteria for Category 1 credit for physicians.

For further information, contact: Extended Programs in Medical Education, University of California School of Medicine, Rm 569-U, San Francisco, CA 94143 (telephone: 415-666-4251).

Diagnosis and Treatment Strategies for Gynecologic Cancer

Houston, Texas, November 13–16, 1985

This 29th annual Clinical Conference sponsored by The University of Texas M. D. Anderson Hospital and Tumor Institute will include comprehensive presentations on the more common endometrial, cervical, and ovarian cancers in addition to such topics as biological response modifier therapy and tumor markers.

For additional information, contact: Office of Conference Services, Box 131, M. D. Anderson Hospital and Tumor Institute, 6723 Bertner Ave, Houston, TX 77030 (telephone: 713-792-2222).

Third International Conference on Malignant Lymphoma: Current Status and Prospects

Lugano, Switzerland, June 10–13, 1987

The Palazzo dei Congressi is the location for this series of lectures and workshops dealing with the diagnosis and treatment of Hodgkin's and non-Hodgkin's lymphoma in adults and children. Most lectures will be given by invited speakers. The program will include free presentations, workshops, and a poster session. The deadline for the receipt of abstracts is *January 15, 1987*.

For further information contact: F. Cavalli, MD, Division of Oncology, Ospedale San Giovanni, 6500 Bellinzona, Switzerland (telephone: 091-23-62-10).

ANNOUNCING A NEW NCI MONOGRAPH

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The most recently published monograph in the JNCI series is now available from the US Government Printing Office. Edited by Madhu A. Pathak and June K. Dunnick, this volume (No. 66) represents the proceedings of a conference held in Research Triangle Park, North Carolina.

The National Toxicology Program, acting on the request of several governmental agencies, initiated a research program for investigators to study the toxicologic properties of psoralen and high-intensity longwave ultraviolet radiation and to gather data on the safety and effectiveness of this mode of treatment.

Subsequently, the Program sponsored this conference on psoralens so that scientists working in this area of research could discuss the photobiologic properties of these compounds; their mutagenicity; and the carcinogenic, immunologic, and analytic aspects of the combination therapy, as well as its side effects, safety, and therapeutic benefits. Also included is a history of the research and development of oral psoralen and longwave radiation photochemotherapy, a treatment that began in Egypt and India thousands of years ago.

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